









## LESIONS PRODUCED BY ARSENICALS AND THEIR BEARING ON THE PROBLEM OF SPECIFIC ARSENIC THERAPY.\*

By WADE H. BROWN, M.D.

*(From the Laboratories of The Rockefeller Institute for Medical Research.)*

The use of arsenic in the treatment of trypanosomiasis is now of some fifty years' standing. The first therapeutic experiments of this nature were those of Livingstone in the treatment of "fly bite" or nagana during his African explorations in 1858. True, he had no knowledge of the causative agent against which his therapy was directed and it was not until many years later, when the trypanosomal nature of nagana, surra, sleeping sickness, and other diseases of a similar character had been established, that the significance of these early therapeutic efforts was grasped and definite progress towards a rational therapy of these diseases was made.

Experimental arsenic therapy had its beginning in the demonstration by Lavéran and Mesnil of the trypanocidal action of arsenic in laboratory animals. This work, published in 1902, inspired a search for specific therapeutic agents and many other lines of therapy began to be developed in consequence—notably dye therapy, and later the use of compounds of antimony. The greatest achievement, however, came from the work of Thomas on atoxyl published in 1905, followed by that of Uhlenhuth extending the field of specific arsenic therapy to spirochætal infections. With the determination of the chemical constitution of atoxyl by Bertheim and Ehrlich, the foundations for the future development of specific arsenic therapy were completed. Under the leadership of Ehrlich, the achievements in this field of therapy during the last decade have indeed been most remarkable. But the end is not yet and there are many work-

ers striving, with hope, to improve upon the results thus far obtained.

During the past several years, a small group of workers at the Rockefeller Institute, consisting of Drs. Jacobs and Heidelberger in the chemical department, and Dr. Louise Pearce and myself, have been struggling with the problem of specific chemotherapy. There are many phases of this work about which one might speak, but we have chosen that phase about which least has been said and which to our minds, at any rate, presents at once the most difficult and the most hopeful point of attack, namely, injury and its elimination.

Since the aim of all efforts to construct chemical compounds possessing specific action against agents of infection is not only to cure, but to cure with a minimum of risk and injury to the host, it is quite obvious that the attention of the experimental worker must be constantly centered upon two points—effect upon the host and effect upon the parasite. These two phases of drug action are our directing forces and are of equal importance. Further, they are the criteria by which we must gauge the value of each product. Intensification of the action upon the parasite and the elimination of injury to the host become, therefore, our constant aim, and I refer here in particular to that phase of injury which gains expression in the form of organic lesions. While innumerable compounds of arsenic must have been investigated from this dual aspect, it is unfortunate that so little of the details of their action upon the animal organism has found a place in the literature. True, the pathology of arsenic poisoning has been most carefully worked out and serves as a valuable guide and standard for comparison but, apart from this, as I shall attempt to show, this knowledge avails but little in the problem of chemotherapy.

With the small group of compounds that we have studied thus far—about sixty in all—it has been our plan to determine not only their toxic limits, but the essentials of their pathological action in mice, rats, guinea-pigs, rabbits, and, in some instances, dogs. The toxicity of compounds with approximately the same percentage of arsenic has varied very widely, but not more so than the variety and severity of the tissue injury they are capable of producing.

Omitting most of the well-known lesions of arsenic, some con-

ception of the complexity of the problem presented to the experimental worker may be conveyed by a brief description of the more important types of lesions produced in a given organ by different compounds of arsenic, the preferential action exhibited by different compounds for different organs, variations of the action in different animal species, and some of the so-called accidents. From these it will not be difficult to grasp the fundamental importance of the lesions of arsenicals to the experimental worker and perhaps also to the clinician.

The pathological changes that one encounters in the heart and blood-vessels constitute a group that are of great importance. Many of these lesions are extremely difficult of demonstration either to the naked eye or with the microscope; some are of the character of degenerative processes in the heart or vessel walls, which in themselves may seem of little importance but furnish a basis for more serious lesions, such as hemorrhage or thrombosis. With some compounds, subendocardial hemorrhages, myocardial hemorrhages, and hemorrhage into the substances of valves, have been practically constant. These lesions are especially striking in the heart of the dog, to which relatively large doses of atoxyl have been administered. They are also known to be produced by such substances as salvarsan and its various modifications.

Vascular occlusion is probably far more common as the result of poisoning with arsenic or compounds of arsenic than is generally recognized. These occlusions may be produced by either thrombi, formation of which is favored by vascular injury, or by emboli resulting from coagulation of blood elements. The seriousness of the injury produced by such vascular occlusions, of course, depends largely upon their extent and location, and all such changes may be regarded, therefore, as accidents. Many of them may be relatively harmless, whereas others, by chance involving tissue of vital importance, may lead to most serious consequences.

Turning now to the lungs, until recently we had observed no lesions of the lungs that could be regarded as both constant and characteristic of the action of any particular compound. We have, however, observed with at least five recent compounds, a characteristic type of lung injury. This injury consists of petechial hemor-

rhages, finely and uniformly distributed through the substances of a lung which is voluminous and pale. With some of these compounds, a lesion of identically the same character is present in the surface of the kidney, while with others the kidney lesion is slight or entirely absent. With at least two of these compounds, reduction of the dose leads to a disappearance of the lesions in the kidney and the lesions in the lungs persist as the one characteristic change produced by the compound.

The lesions of the liver have long ranked among the most important of the organic changes produced by arsenicals. For convenience, we may divide the lesions of this organ into three groups: first, the lesions of a diffuse character, showing no preference for any particular region or structure of the organ; secondly, those lesions produced by compounds manifesting preference for the central zone of the liver lobule, and thirdly, the lesions produced by compounds acting by preference upon portal structures and the peripheral zone of the lobule.

The lesions in the first group are, for the most part, degenerative in character and closely resemble the well-known changes produced by inorganic compounds of arsenic. To these, however, must be added instances of diffuse necrosis, in which the maximal action of the compound upon the liver manifests itself in a widely distributed necrosis of individual liver cells; finally, we may place here also the group of focal necroses which are quite frequent and characteristic of the action of many compounds of arsenic.

Lesions of the central zone also include degenerative processes and necroses. Fatty degeneration of the central zone of the liver lobule is quite frequent, and in some instances the most constant and striking lesion produced by certain compounds. In other instances, fatty degeneration gives place to necrosis and most of these central necroses differ in no wise from the central zone necrosis produced by a large number of chemical agents.

We have, however, one compound on our list that produces a series of lesions entirely different from any lesion-complex with which we are familiar. The action of this compound is characterized by the fact that lethal doses in the guinea-pig lead to profuse hemorrhage into the bile-ducts from which the animal may actually bleed to

death. At autopsy the appearances presented are those of a nutmeg or extremely pale and pasty liver with a gall-bladder enormously distended with blood, while the kidneys are extremely pale throughout and the urinary bladder is distended with an almost water-clear urine. We have not found this lesion-complex so constant for any other animal. It has been observed but once in the rabbit, and we have failed entirely to produce it in the dog, which may be explained by the fact that the dog occupies an anomalous position with reference to this compound, in that dogs tolerate more than twice as much of this particular arsenical than any other animal upon which we have tested it. This is, so far as we know, the only instance in which the dog tolerates more of an arsenical than other experimental animals. It is also of considerable interest that this particular compound is one very closely related to atoxyl, a substance to which the dog manifests an extreme degree of susceptibility, tolerating only 10 mg. per kilo of body weight, as against 170 mg. for the mouse or rat.

The repair that follows this type of central necrosis in the guinea-pig is also quite peculiar. The liver of the pig that has recovered from a single large dose or from repeated smaller doses of this substance is a hob-nailed liver. This hob-nailed appearance seems to result from some degree of connective-tissue increase about the central and sublobular vessels with resulting contraction in these territories. It is, however, helped out by the fact that the liver cells are extremely large and hydropic, producing the effect of a swelling in the liver parenchyma. These changes are also associated with peculiar vacuolations that are particularly numerous about the central and sublobular vessels but are also present in other portions of the lobule. These spaces are filled, for the most part, with albuminous material, though they occasionally contain cellular detritus or a few red cells. Ascites is usually present.

In contrast to this group of central lesions, there are numerous substances that affect, by preference, the structures of the portal space and the periphery of the lobule. Most of these show a common type of action. In large doses, they produce complete necrosis of all structures in the portal space with a surrounding zone of liver cells. If the dose be reduced, however, the structures which mani-

fest the greatest degree of injury are the bile-ducts. With even a small dose, a high degree of necrosis of bile-duct epithelium results while there is only a slight degree of fatty degeneration of the peripheral liver cells. Repair of lesions of this type takes place rapidly and is manifested by numerous mitoses in the bile-duct epithelium with an outgrowth of this epithelium toward the liver tissue and by marked proliferation of the connective tissue of the portal space. From this type of injury there results, therefore, a perilobular fibrosis which apparently interferes in some measure with the normal liver functions, as these animals always manifest a high degree of jaundice and frequently a pronounced ascites. In animals that recover from the early effects, some degree of absorption of connective tissue undoubtedly occurs, but the bile-ducts in the residue of this connective tissue are usually profuse, tortuous, and markedly dilated, indicating the persistence of some degree of bile-duct obstruction.

It is interesting to note in connection with lesions of this type that the most pronounced effect of the drug is produced by the first injection and that subsequently the dosage may be increased, even above the usual lethal limits, without materially altering the effects of the initial injection. Apparently, the tissue of this injured territory, once regenerated from previously injured cells, acquires a distinct degree of tolerance, or tissue fastness, and is not easily injured by further use of the drug. It is possible, however, to increase the dose until necroses again result. When such necroses are produced, however, they no longer involve the portal tissues or peripheral zone of the lobule but are localized beyond this territory, in the mid-zone of the lobule, and we are inclined to interpret this shifting of the point of injury as further suggesting a fastness of portal and peripheral tissues.

While speaking of the subject of fastness, it is perhaps well to call attention to the reverse of this phenomenon, namely, tissue sensitization. Many of the compounds that produce these peripheral lesions are compounds that also produce some degree of focal necrosis, and we have observed with some of them that, whereas a fastness of peripheral tissues may be produced by repeated injections, after a lapse of two or three weeks, the injection of a dose that previ-

ously produced scanty, if any, focal necrosis, will result in the production of innumerable such necroses. We know of no explanation that can be offered for this enormous increase in the focal effects of such compounds except to suggest a "sensitization" to this particular phase of the drug action.

Turning now to the kidney, this is the organ about which the greatest interest in the action of arsenic has centered, and one of the recognized types of experimental nephritis is that produced by the administration of arsenic. We have, however, not a single type of the arsenic kidney, but certainly two very sharply differentiated types with innumerable variations or gradations. The classical arsenic kidney, as is well known, is one that grossly may be described as a red kidney. On section, it exudes blood and shows a diffuse reddening from the outermost edge of the cortex to the pelvis, with little, if any, preferential action manifested in any of the zones of the kidney. There are numerous compounds of arsenic, the lesions of which conform more or less to this general picture. Some of these compounds, however, produce a greater degree of necrosis of tubular epithelium, while others exhibit a preferential action as regards the vascular changes, some producing the most marked alterations in the cortex, others in the medulla and still others showing hemorrhage and congestion confined almost entirely to the boundary zone. In some of the latter group, increase in the dose of the drug causes hemorrhage and congestion to spread through the cortex, while with others the extension is in the opposite direction.

A sharp contrast to the red arsenic kidneys is shown in a group of typically and extremely pale kidneys. With this group, there may be no evidence in the gross appearance of the organ of either hemorrhage and congestion to spread through the cortex, while with is involved to a very high degree. Connecting this group of pale kidneys with the typical red kidneys, however, comes a group of pale kidneys in which hemorrhage to a greater or less degree appears in the boundary zone. For example, arsacetin produces typically pale kidneys when administered intravenously in an amount sufficient to kill a dog in 24 hours. Atoxyl, when given in as small a dose as 20 mg. per kilo of body weight, produces a distinct and narrow line of hemorrhage in the boundary zone of an organ that is otherwise



quite pale. As this dose is increased up to the lethal limits, this line of hemorrhage spreads until it involves the entire medulla, converting it into what is essentially a blood clot. The hemorrhage also shows a tendency to spread outward, but to a far less degree, and usually the outer surface with some depth of subjacent cortex is distinctly pale, while the tubular epithelium shows marked necrosis.

Before leaving this subject, it is interesting to note also that the chronic lesions produced by these compounds of arsenic are localized and characterized according to the nature and seat of the lesions of acute poisoning. For example, compounds that produce acute lesions of the boundary zone are usually characterized in chronic stages by marked proliferation of connective tissue of this zone which, of course, may lead to secondary changes in the structures, both above and below. In all these types of renal injury, a remarkable degree of uniformity in the nature and seat of the lesion produced by a particular compound has been observed. These lesions, however, are susceptible to a certain degree of modification depending upon the size of the dose, the rapidity of action of the compounds and the length of life of the animal subsequent to the administration of the drug.

Another group to which we are inclined to attribute a great deal of importance is that of the lesions of the adrenals. The actual importance to be attributed to lesions of these organs is, of course, intimately related to their functional integrity. In the second place, no notice seems to have been taken of injury to the adrenal as a result of arsenical intoxication. We have found, however, that some degree and some type of adrenal injury are among the most constant changes produced by all classes of arsenicals. We may consider the evidences of injury from the standpoint of alterations in the lipid content, structural changes in the cortex, structural changes in the medulla, and variations in the chromaffine content. All arsenicals that we have tested cause alterations in the physical character of the lipoids in the cortex of the guinea-pig. The fine droplets in which this lipid usually exists, give place to larger droplets, especially at the outer and inner edges of the waxy cortex. The lipid which is usually abundant in this zone then begins to decrease in the middle of the zone, but increases in both the zona glomerulosa and the

pigmented zone of the cortex. With some arsenicals, this decrease and shifting continues until the waxy cortex may be practically free of all lipoids except at its extreme outer and inner edges; and finally, there may be no lipoid in the cortex except at the outer edge of the zona fasciculata. Corresponding to these changes in the nature and distribution of the lipoid we find a greater or less degree of degeneration, necrosis and disintegration of the cells of the adrenal cortex, and with some compounds hemorrhage is a prominent feature of the structural change. Regeneration of cortical cells after injury with most compounds is quite rapid, however, and numerous cells undergoing mitosis may be observed, especially in the outer half of the zona fasciculata.

In the medulla of the adrenal, the changes are equally striking; the most striking are perhaps those involving the chromaffine substance. While with some compounds of arsenic we have noted but little, if any, alteration in the chromaffine content, other compounds produce a very marked decrease in this substance. With some, the disappearance of chromaffine takes place early and quickly with rapid regeneration, while with others the early changes are but slight and it is only after some days that a distinct reduction in the chromaffine can be demonstrated. Another change observed in the medulla is of the nature of a round-celled or polyblastic infiltration, usually associated with some degree of endothelial and connective-tissue hyperplasia. The significance of this last group of changes is still uncertain, as we have observed similar changes, but of a less degree, in the medulla of animals after acute poisoning or even in controls. As I have said, injury to the adrenals seems to be common to all arsenicals, but here again it must be emphasized that the type and degree of injury vary very decidedly with different compounds and different animal species; and it has appeared to us that the measure of injury inflicted upon the adrenal by a particular arsenical was parallel with the susceptibility of a given animal species for a given compound.

The lesions described in these several organs can serve to give only a general conception of the diversity of lesions produced by different compounds of arsenic. The changes in other organs are equally varied and of just as great importance in their practical bearing, especially those of the nervous system and of the blood and

blood-forming organs, the details of which cannot now be presented. One group of changes in connection with the pancreas may be cited, however, to emphasize the question of animal specificity. We have in our list of compounds a number of substances that produce widespread fat necrosis as a part of their characteristic action in mice and rats. With none of these substances, however, have we been successful in producing fat necrosis in guinea-pigs, only once in a rabbit and a few times in the dog, but in no instance was fat necrosis a constant manifestation of the drug action in any animal species except mice and rats.

So much for the lesions themselves which, undoubtedly, are of great importance in their bearing on pathological problems, but this is not the point from which we wish to consider them.

As I have previously indicated, injury is one of the standards by which we must gauge the value of our products and it is easily seen that upon this basis many compounds can be eliminated, at once, from the list of therapeutic agents, either on account of the seriousness of the injury produced by any or all doses of therapeutic quality, or because of irregularities in their action in different animal species, or of the frequency of irregularities or accidents that may follow their use in any species. Fortunately, there are other compounds of a more hopeful character, and these require close inspection. In determining the therapeutic value of such compounds the attempt has been made to reduce this entire relationship to a numerical basis and to express the therapeutic value as the relation of the

*dosis curativa* to the *dosis tolerata* or  $\frac{C}{T}$ . In actual practice, such expressions are of but little value and are quite misleading, as neither of these values represents constants nor anything approaching a constant in most cases. Strictly, the *dosis tolerata* has been defined as the dose that evokes neither lesions nor symptoms, but in practice this standard has not been rigidly adhered to and the values usually given are hardly more than survival doses. In truth, it is doubtful if there is, as yet, a single compound of arsenic, possessing a *dosis curativa* against experimental trypanosomiasis that produces neither symptoms nor lesions.

Further, such a basis of valuation implies a limitation of thera-

peutic standards to the efficacy of single dosage, or the method of *therapeia magna sterilisans*, and we are by no means prepared to concede that this is the only system of therapy or even the method of choice in all cases. The point of vital importance in determining the therapeutic value of arsenicals is the degree of injury that may follow curative applications of a given compound by whatever system it may be necessary to employ such a compound.

In weighing the pathological data, therefore, it is necessary to grasp clearly the relation between the method of employing the drug and the resultant injury, the difference between the characteristic lesions and the accidental lesions and the limitations fixed by these to the manner in which a given drug may be used. For example, there are compounds of arsenic which may be used in the theoretically curative dose with but slight recognizable injury. The usefulness of some of these compounds, however, is decidedly limited by the fact that repetition of even smaller doses may be attended by a pronounced accentuation of the injury, and in some instances an apparent sensitization occurs, giving rise to an increase in the frequency of accidental lesions produced by such compounds. On the other hand, some arsenicals permit of frequent repetition and even an eventful increase in the dose above the usual lethal limits with little, if any, increase in the injury produced by a single large dose. Instead of a dominant sensitization, we have here a rapidly developing tolerance or tissue fastness which tends to broaden the scope of usefulness of such compounds. Judged by the criterion of injury, therefore, the compounds of choice are those that manifest the most constant toxic action in all species of animals and permit of the greatest latitude of usage with a minimum of injury.

Since, from a constructive standpoint, the elimination of injury to the host is of prime importance, it follows, from what has gone before, that we must distinguish between toxicity and tissue injury, because it now appears that one may be diminished without necessarily reducing the other. It seems, indeed, that each compound of arsenic is capable of setting up a lesion-complex which is definite, although more than one compound may produce nearly identical lesion-complexes; in the complexes, moreover, the lesions of one or possibly two organs are dominant. This being true, it follows that

the lesion-complex is measurably related to the chemical constitution of the compound, from which fact it follows further that the determination and study of the lesion-complexes must inevitably influence the construction of therapeutically active drugs. But our present knowledge of how to remove the defects of chemical compounds is limited in the extreme. The outlook is hopeful, however, that as clinical and experimental experiences increase in number and precision, a rational basis of procedure may emerge.

In conclusion, in presenting this seemingly unpopular phase of specific arsenic therapy, the subject has been considered entirely from the view-point of the experimental worker. The achievements in this field of work have been truly marvelous and we must emphasize the fact that we do not wish to detract, in any way, from the value of these achievements to clinical medicine nor to create any unfavorable impression against arsenic therapy. On the contrary, we wish merely to emphasize the importance of a thorough knowledge of the lesions produced by compounds of arsenic in all their bearings as a means to an end, namely—the successful prosecution of the problem of specific arsenic therapy.

## BACTERIOLOGICAL AND CLINICAL STUDIES OF AN EPIDEMIC OF KOCH-WEEKS BACILLUS CON- JUNCTIVITIS ASSOCIATED WITH CELL INCLUSION CONJUNCTIVITIS.<sup>1</sup>

BY HIDEYO NOGUCHI, M.D., AND MARTIN COHEN, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research and  
the New York Post-Graduate Medical School and Hospital, New York.)

PLATES 41 TO 43.

(Received for publication, June 21, 1915.)

While engaged in the study of conjunctivitis associated with epithelial cell inclusions, the occurrence of an epidemic<sup>2</sup> of conjunctivitis due to hemoglobinophilic bacilli enabled us to investigate the relationship between this organism and the epithelial cell inclusions.

Since the discovery of the inclusions by von Prowazek and Halberstaedter, who considered them to be the etiological agents of trachoma, an abundant literature has arisen. Opinions are still greatly divided as to the nature of the bodies, although the majority of investigators regard them as an independent organism, as claimed by von Prowazek and Halberstaedter. They do not, however, commit themselves to the statement as to whether the organism represents the causative agent of trachoma or not. We were inclined to the belief that it constitutes an independent organism which produces a true conjunctivitis of a more or less characteristic clinical course. Herzog<sup>3</sup> considered them to be mutation forms of the gonococcus, while Williams<sup>4</sup> views them as the cell inclusions of various organisms, such as the hemoglobinophilic bacillus and allied species.

<sup>1</sup> Read before the Ophthalmological Section of the New York Academy of Medicine, May 17, 1915.

<sup>2</sup> Service of Dr. Martin Cohen at the Randall's Island Hospital, New York.

<sup>3</sup> Herzog, H., *Arch. f. Ophth.*, 1910, lxxiv, 520.

<sup>4</sup> Williams, A. W., *Collected Studies from the Bureau of Laboratories, Department of Health, City of New York*, 1912-13, vii, 159-247; *Jour. Infect. Dis.*, 1914, xiv, 261.

Although by means of a special method an organism strikingly similar to the epithelial cell inclusions has been made to grow in pure culture,<sup>5</sup> their pathogenic properties, nevertheless, have not yet been established.

As a result of our previous studies a tentative conclusion had been reached that infection of the conjunctiva with these organisms produces an independent conjunctival disease which may properly be described as cell inclusion conjunctivitis. This organism may be present alone, or it may be associated with other pathogenic organisms.

### *Outbreak and Course of the Present Epidemic of Koch-Weeks Bacillus Conjunctivitis.*

Prior to the outbreak of the Koch-Weeks bacillus epidemic,<sup>6</sup> one of us (Cohen) had under observation for about five months 10 cases of inclusion conjunctivitis and 4 cases at the outdoor department of the Post-Graduate Hospital. In these cases, smears (and in a few cases, cultures) were taken weekly and examined (Noguchi), but no Koch-Weeks bacilli were found. On October 3, 1914, one of the inclusion cases under observation at the Island developed an acute conjunctival inflammation. In the conjunctival smears numerous Koch-Weeks bacilli, as well as epithelial inclusions, could be demonstrated. The acute conjunctival inflammation spread to the remaining nine inclusion cases. The smears from all showed inclusions as well as numerous Koch-Weeks bacilli.

In an adjoining ward there were under treatment 15 cases of follicular conjunctivitis, 3 cases of interstitial keratitis, and 1 case of chronic dacryocystitis. 13 of these contracted the Koch-Weeks bacillus infection. During the entire course, which lasted from two weeks to seven months, no pathogenic organisms but the Koch-Weeks bacilli could be demonstrated in the smears and cultures.

There were 7 patients in the trachoma ward. 2 of these showed simultaneous presence in the conjunctiva of inclusion and of Koch-

<sup>5</sup> Noguchi, H., and Cohen, M., *Arch. of Ophth.*, 1914, xliii, 117; *Jour. Exper. Med.*, 1913, xviii, 572.

<sup>6</sup> In the present article the hemoglobinophilic bacilli found in these cases were designated as Koch-Weeks bacilli, in order to conform with the usage of this term in ophthalmology and bacteriology when speaking of this class of organisms.

Weeks bacilli. The latter organisms v as there were possibly too few to be recognized in the smears. This condition was present for a short time during this epidemic. The infection with the Koch-Weeks bacillus apparently produced no change in the clinical picture of these trachoma cases. The 4 inclusion cases not treated on the Island showed at no time during the past year the Koch-Weeks bacillus, either in smears or cultures; not even during a relapse or reinfection, of which one of these cases had two, could they be demonstrated. Table I shows the bacteriological findings in the cases on which the present report is based.

TABLE I.

Organisms present,	No. of cases.
1. Epithelial cell inclusions alone .....	6
2. Epithelial cell inclusions at first, with subsequent Koch-Weeks infection	17
3. Koch-Weeks bacillus alone .....	13
4. Koch-Weeks bacillus at first, with subsequent appearance of inclusions	2
5. Pneumococcus with inclusions .....	1
Total .....	39

In acute catarrhal conjunctivitis it is at times difficult, or impossible, to determine the causative organism from the clinical manifestations alone. This is due to the great variability in virulence of the usual infecting organisms, and to the resisting qualities of the individual patient. Yet in many of the acute infections of the conjunctiva, the etiological diagnosis as made by the clinical appearance could be confirmed subsequently by the bacteriological diagnosis, especially in the subacute state. In mixed infections, such a diagnosis is much more difficult.

In a few instances, even smears and cultures of the conjunctival scrapings fail to clear up the diagnosis, as no pathogenic organisms can be found. That the microscope alone is not sufficient to exclude the presence of an organism is shown by the fact that the scrapings from cases of trachoma which showed neither inclusions nor the Koch-Weeks bacilli (as proved similarly by cultures), when inoculated on the conjunctiva of the higher apes (baboons) produced symptoms similar to those which are seen in inclusion cases in man. They were, however, of a milder type and lasted only about ten days. The conjunctival scrapings from the inoculated animals re-



vealed the presence of epithelial inclusions, but no Koch-Weeks bacilli or allied organisms appeared in the smears or in the cultures.

We shall now discuss the clinical manifestations in the group of cases mentioned in Table I.

The clinical course of inclusion conjunctivitis has already been described by Cohen.<sup>7</sup> In the early stage, the clinical manifestations resemble those seen in the Koch-Weeks bacillus infection, and it is only by the examination of smears or cultures that the diagnosis can be positively made. Briefly, they are as follows: In the beginning there is moderate edema of the lids, with mucopurulent secretion. Somewhat later isolated reddish translucent follicles appear in the lower palpebral conjunctiva and upper folds, these two sites becoming involved simultaneously. At a later stage, the upper palpebral conjunctiva takes on a brick red color and assumes the characteristic granular appearance. These regular and progressive manifestations of the disease retrogress after about two months by absorption of the contents of the follicles and papules. These disappear first from the upper portions of the conjunctiva, when the conditions resemble those of cases of follicular conjunctivitis; and later, in about three months, they begin to disappear from the lower conjunctiva. At the end of four months, the conjunctiva is again in a normal condition.

This is the usual course, but at times there are deviations, due to external or unknown conditions, or to a previous affection of the conjunctiva; as, when grafted on a follicular conjunctivitis, the original follicles become larger and new ones make their appearance. In the stage of retrogression the papules become absorbed and the original follicles, owing to relaxation of surrounding pressure, again become prominent, the course being then one of a follicular conjunctivitis. In 2 cases of inclusion conjunctivitis, there remains solely, at the end of nine months, a fine papillary condition of the upper tarsal conjunctiva; one of these two cases has had two relapses or infections.

It may be mentioned here in passing, that of the 75 cases of inclusion conjunctivitis studied by us during the past five years, 58 cases are still under observation of Dr. Cohen. 2 of these still show a diffuse and linear cicatrization of practically the entire palpebral conjunctiva, but no corneal or other involvement is present. 2 other cases of this group still show a fine papillary hypertrophy of both tarsal conjunctivæ, with no corneal or other complications. The remaining 54 cases have remained normal for the past four and a half years. 17 cases were observed for six months, and were normal when last seen.

4 patients with follicular conjunctivitis became infected with inclusions. The follicles previously present became enlarged and congested, and new papules and follicles appeared on the upper tarsal conjunctiva and the conjunctiva of the lower lid. These cases had the appearance of a severe type of inclusion conjunctivitis.

For several weeks after the beginning of the disease, the smears in all cases showed an abundance of epithelial cell inclusions. Toward the end of the disease, the number of inclusions gradually diminished. Yet after three months' duration, although no inclusions were demonstrable in the smears, the clinical aspect of the disease was usually still evident.

<sup>7</sup> Cohen, M., *Arch. of Ophth.*, 1913, xlii, 29.

On Oct. 23, 1914, one of the inclusion cases on the Island developed a slight conjunctival inflammation, which smears and cultures showed to be due to the Koch-Weeks bacillus. In the succeeding two weeks, as previously mentioned, additional cases of inclusion conjunctivitis contracted the Koch-Weeks bacillus infection. Conjunctival smears and cultures examined weekly for the past several months showed at first the simultaneous presence of inclusion and Koch-Weeks bacilli. In the succeeding two months, the findings varied, at times only one of the two organisms being present, at other times, both. Possibly this was due to relapse or reinfection. In the next two months, although no organism was present in smears and cultures, there was still evidence of inflammation. Ultimately the conjunctiva became normal in all but one case. In this case there is still at the end of a year evidence of an inclusion conjunctivitis in the retrogressive stage.

In an adjoining ward, where mild follicular conjunctivitis cases were being treated, 13 contracted the Koch-Weeks bacillus infection. Previous to the Koch-Weeks infection, all these cases showed over various areas of the conjunctiva follicles varying in number and size, and showing no inflammatory reaction. The first case in this ward developed on Nov. 1, 1914. Only 3 of the 16 cases escaped the Koch-Weeks infection. The Koch-Weeks bacillus was present in great numbers, but no other pathogenic organisms could be demonstrated throughout the entire course of the epidemic, which lasted six months.

The clinical manifestations in these follicular cases were as follows. There were slight edema of the lids and conjunctival congestion with mucopurulent secretion. Both eyes became successively involved. The acute infection lasted six to eight weeks. At the end of this period, the original follicles were still present.

In one of the typical follicular cases it was difficult to determine whether the acute infection was due to the Koch-Weeks bacillus or to the epithelial inclusions. The symptoms were those of a severe inclusion conjunctivitis. But notwithstanding repeated examinations extending over two months, no inclusions could be found. Nevertheless, judging from the clinical course and from the fact that cell inclusions are often absent in the subacute stage, this and similar cases may be considered due to epithelial inclusions.

2 cases of interstitial keratitis on the Island Eye Ward and 1 at the Dispensary had normal conjunctivæ, when they suddenly developed an acute catarrhal conjunctivitis. In the smears and cultures, which were taken weekly for four months, the Koch-Weeks bacillus was present, even at a time when the conjunctiva was free from inflammation. The inflammatory stage lasted from six to eight weeks. Inclusions could at no time be demonstrated in the smears.

The clinical appearance in these cases was moderate edema of the lids, and conjunctival congestion with mucopurulent secretion. In one case there was present a small scleroconjunctival hemorrhage with a small phlyctena. Conjunctival furrows or folds also appeared in those cases when the congestion diminished.

As regards the last group in the table, 2 cases at first showed the Koch-Weeks bacillus in the conjunctival smears. Later, inclusion cells made their appearance, associated with their clinical manifestations. In the first case there was present

a mild catarrhal conjunctivitis, papules being present on the lower tarsal conjunctiva alone. In this respect it differed from other inclusion cases where the papules are present also on the upper tarsal conjunctiva. It is possible that the lesions at times can only be detected microscopically. A second examination in this case revealed the epithelial inclusions. Perhaps the inclusions were missed in making the first examination, as it is likely that in some stages of the disease only a few epithelial cells contain inclusions.

The second patient originally had a follicular conjunctivitis. He then contracted an acute catarrhal conjunctivitis, the secretion containing for three weeks the Koch-Weeks bacillus alone. At the end of this time, when the conjunctivitis improved, he again developed an acute conjunctival inflammation, and the smears showed numerous inclusions and a few Koch-Weeks bacilli. This case then followed the usual course of an inclusion conjunctivitis, the conjunctiva ultimately returning to a normal condition.

In a ward assigned to minor eye affections, where there were a few mild Koch-Weeks cases, a routine examination of the conjunctivæ of all the cases was made, in order to determine the presence of the Koch-Weeks bacillus. 2 cases showed the Koch-Weeks bacilli, in spite of the fact that the conjunctivæ were entirely normal throughout the whole period. In another case in the same ward degenerated Koch-Weeks bacilli were found in the conjunctival smears. This case then developed an acute inflammation, showing all the symptoms of an inclusion conjunctivitis with the added findings of epithelial cell inclusions in the smear examination.

In our routine examinations made during the past five years Koch-Weeks bacilli have rarely been found. Recently we encountered eight cases of pneumococcal conjunctivitis. Clinically these cases resembled acute catarrhal conjunctivitis, due to the Koch-Weeks bacillus, and were so diagnosed before the smears and cultures showed the presence of numerous pneumococci without any Koch-Weeks bacillus. The conjunctival secretions in these cases were typically serous, being also associated with diffuse congestion. The duration was from one to two weeks. One of the patients, included already in this paper, had originally an inclusion conjunctivitis. Later, he contracted a Koch-Weeks bacillus infection. After four weeks, when apparently cured, another acute inflammation appeared, which was due to the pneumococcus. This infection was followed by the appearance of inclusion conjunctivitis, the smears showing both pneumococcus and inclusion cells. At present, the clinical symptoms are those of an inclusion conjunctivitis in a state of relapse or reinfection.

The treatment adopted in all these cases was irrigation of the conjunctival sac with a saturated solution of boric acid every two hours, and the application to the conjunctiva, once a day, of a silver nitrate solution, until the acute symptoms had subsided, when irrigation alone was continued. This method of treatment had practically but little effect on the course of the inclusion cases, whereas in the Koch-Weeks infections decided improvement was observable. The disappearance of the organisms from the smears and culture was, however, not at all influenced by the application of the silver nitrate.

*Bacteriological and Experimental Studies of the Present Epidemic.*<sup>8</sup>

Our technique for obtaining smears and cultures was as follows. The upper lid was everted and then the short end of a sterile slide was gently rubbed over the upper tarsal conjunctiva where there is least likelihood of contamination. By means of a platinum loop, a part of the scrapings removed was used for the purpose of cultivation and animal experiments. The remainder of the materials on the slide was transferred to another slide where a thin spread was made, and examined by means of the Giemsa or Gram stain.

Since our problem deals with the Koch-Weeks infection, the culture media employed were chiefly blood agar and tissue ascitic fluid. The cultural findings are summarized below.

In the present epidemic it was noticed that various strains of the so called Koch-Weeks bacilli or hemoglobinophilic organisms in cultures varied in size to such an extent that they may be divided into a thin (Figs. 4 to 7), a medium (Figs. 8 to 11, 20 to 23), and a coarse type (Figs. 16 to 19), according to their morphology. The coarse variety were bacillary or coccoid (Figs. 12 to 15) and resembled culturally and morphologically some strains of *Bacillus influenzae* (Figs. 24 and 25) derived from the respiratory organs; while the thinner variety was much narrower and often shorter. After several days' cultivation at 37° C. on blood agar, some of the organisms became somewhat granular and unevenly stainable; when found in large masses they often assumed the appearance of the granular forms of the so called trachoma bodies (Figs. 5 to 7, 9 to 11, 13 to 15, 17 to 19). But these granules did not break down so as to approach the minuteness of the elementary bodies (Figs. 32, 33, 36, 39, 43, 45) which are found in the uncomplicated cases of inclusion conjunctivitis or trachoma. The degenerated or involuted bacilli do not have the sharp contour of the elementary granules. Transplants made from these somewhat degenerated granular masses of the hemoglobinophilic organisms give a good growth of typical bacillary or semi-coccoid forms. The character of the colonies on the blood agar is also distinctive, in that they remain minute, sharply elevated with a pointed top, and appear more grayish than the colonies of the

<sup>8</sup>This part of the work was carried out at The Rockefeller Institute.

influenza type varieties, which show a tendency to spread and are of a more dewy aspect. It is quite possible that the minute type constitutes a group by itself, but further study is required to determine its relation to the other groups. At all events several strains of influenza bacillus<sup>9</sup> derived from cases of meningitis and pneumonia appear quite different from the conjunctivitis strains in their morphological features (Figs. 24 to 28). The frequency with which organisms of different type were found in the present epidemic is shown in Table II.

TABLE II.

Organisms present.	Types.		
	Coarse.	Medium.	Thin.
1. Epithelial inclusions first, with subsequent Koch-Weeks infection (17 cases) .....	1	7	9
2. Koch-Weeks bacillus alone (13 cases) .....	1	10	2
3. Koch-Weeks bacillus first, with subsequent appearance of inclusions (2 cases) .....	1	1	
	(round type)		

As will be seen from Table II, the thin variety occurred very frequently in the cases in which the patients had been previously infected with the inclusions. In the simple Koch-Weeks cases the medium was predominating. It is significant that in the smears taken from the cases infected with the inclusions and Koch-Weeks bacilli it is not difficult to differentiate the initial bodies of the former from the bacilli, because the initial bodies are much larger and oval in shape, and take up a deeper blue stain. Fig. 3 shows a typical inclusion near the nucleus and some Koch-Weeks bacilli in the same cell. The hemoglobinophilic bacilli are seen to be taken up by the polynuclear leucocytes more frequently than by the epithelial cells (Fig. 1), while the inclusion bodies are chiefly epithelial and seldom leucocytic in nature (Figs. 29 to 45). So far as could be ascertained in stained specimens the bacilli remain well preserved within the cells and do not seem to disintegrate into granules (Fig. 2). On the other hand, the initial bodies are often ill defined and do not show any distinct bacillary forms. They are far more

<sup>9</sup> We are indebted to Dr. Martha Wollstein for these strains, for which we here wish to express our thanks.

pleomorphic than the hemoglobinophilic bacilli (Figs. 29, 34, 37, 40, 41, 43, 46, and 47).

The isolation of the hemoglobinophilic organisms, irrespective of the type, was easily accomplished by means of the blood agar; but in many instances the preliminary microscopical search in the smears failed to demonstrate the presence of a few organisms, until they could be found by the cultural procedure. It must be understood that in cases where the inclusions only were found, repeated efforts to isolate the hemoglobinophilic organisms were made, with, however, invariably negative results. In this connection it may be added that a number of cases clinically diagnosed as trachoma was also studied in order to see if any hemoglobinophilic organism could be isolated. The results were uniformly negative. A few cases were also found in the same wards, which may have been infected through contact with the inclusion cases and which, although carefully observed from the earliest stage of the disease, did not at any period show the presence of any hemoglobinophilic bacilli.

*Transmission of the Inclusion Bodies to Animals.*—In order to find out whether the inclusion virus ever exists in the form of a hemoglobinophilic bacillus, scrapings from the conjunctivæ of these cases were inoculated into the conjunctiva of a baboon. Prior to the inoculation the conjunctiva of the baboon was examined to ensure the absence of such a bacillus. Within seventy-two hours after the inoculation the conjunctiva showed moderate congestion, edema, and a few minute papules; a small quantity of a mucopurulent discharge was present at the inner canthus. The smears and culture were made from the conjunctival scrapings. The result showed that there were numerous cell inclusions, but no hemoglobinophilic organisms<sup>10</sup> (Figs. 46 to 51). The examinations were continued regularly for a period of two weeks and the results were unvarying. As was previously shown by various investigators, the von Prowazek bodies are transmissible to higher apes, but the hemoglobinophilic or Koch-Weeks bacillus is not.

*Attempts To Produce Koch-Weeks Bacillus Conjunctivitis in Animals.*—Several attempts were made to transfer the hemoglobino-

<sup>10</sup> Cultures made after 24 and 48 hours were also negative in regard to the hemoglobinophilic bacillus.

philic organisms to the conjunctivæ of rabbits (young and adult) and monkeys (baboon and several *Macacus rhesus*), by introducing several loopfuls of the twenty-four hour blood agar cultures of different strains into moderately abraded surfaces of conjunctivæ. The results were completely negative, except in the case of one rabbit, where the organism was still recoverable after twenty-four hours. It seems remarkable that such a large quantity of pure cultures of freshly isolated strains of these organisms from the cases where the inclusions were also present should fail to reproduce the conjunctivitis, in view of the fact that a comparatively small number of the inclusions as contained in the scrapings from a patient can readily reproduce the inclusion conjunctivitis.

From this a conclusion may be warranted that in a conjunctivitis where the inclusion and the hemoglobinophilic bacilli are simultaneously present, two pathogenic factors can be separated by means of transmission of the material into the conjunctiva of a suitable animal (baboon) in which the inclusion virus alone implants itself upon the new host, while the bacilli quickly disappear from the inoculated conjunctiva. For man both organisms are pathogenic, but for the baboon only the inclusion virus is capable of producing infection.

On the other hand, this may not exclude the possibility, as asserted by Williams and her associates, that a conjunctivitis due to the Koch-Weeks bacillus may also show some cell inclusions; since under certain experimental conditions a very suggestive phenomenon, to be related below, has been observed.

*Attempts To Produce the Koch-Weeks Bacillus Epithelial Inclusions in Animals.*—In order to determine experimentally whether or not the hemoglobinophilic bacilli when taken up by epithelial cells will undergo the morphological changes which lead to the formation of so called inclusion bodies, an intratesticular inoculation of the rabbit with pure cultures of the hemoglobinophilic organisms isolated from the cases already mentioned was resorted to. The local reaction which follows consists of edema of the scrotum and induration of the testicular parenchyma within twenty-four hours. The edema gradually disappears within the next few days, while the testicular induration remains more or less the same for about five days, after which it commences to recede. In some instances the

rabbit succumbed to septicemia and probably to intoxication as a result of the introduction of the cultures. The organs were removed at intervals of twenty-four hours, three days, and six days, and then fixed in sublimate alcohol, and stained by Giemsa's acetone method,<sup>11</sup> and, if overstained, treated with a 10 per cent solution of *Glycerin-äthermischung* (Grübler) for a few minutes, as advocated by one of us.<sup>12</sup> The results show that the injection of the bacilli is followed by an intense leucocytosis, in which the polynuclears invade the tubules in groups. In the twenty-four hour specimens the organisms are still well distributed along the interstitial spaces; in the three day specimens one notices numerous masses of agglutinated bacilli here and there within the tubular lumina or along the connective tissue. These masses take on a purplish hue and appear granular in structure and indefinite in outline. They are on the point of disintegration. The granules within and about these bacterial masses are not so minute as to be mistaken for the elementary bodies of the inclusion. In the six day specimens some clumped bacteria were found within the polynuclear leucocytes, but a diligent search failed to show any typical epithelial cell inclusions. In these six day preparations the number of the bacterial clumps is smaller than at an earlier period. There were no granules small enough to be regarded as the typical elementary granules. While a careful comparison of the inclusions and the bacterial clumps just referred to will reveal the difference between them, this is not always easy to accomplish (Figs. 53 to 55). Fig. 52 shows a mass of granules from a case of mixed infection of Koch-Weeks bacilli and the von Prowazek inclusions, and it appears difficult to determine whether they represent the degenerated bacilli or the inclusion granules. The deep stain of the mass and the absence of any free bacilli around it seem to indicate that it belongs to the latter kind. It is also possible that some of the clumped granules found in the conjunctival smears from cases of Koch-Weeks infection might have been interpreted as the cell inclusions and classified with the von Prowazek inclusions.

*Attempts To Transmit the Inclusion Bodies to a Parenchymatous Organ in Animals.*—Efforts were also made to transmit the von

<sup>11</sup> Giemsa, G., *Deutsch. med. Wchnschr.*, 1909, xxxv, 1752.

<sup>12</sup> Noguchi and Cohen, *Proc. N. Y. Path. Soc.*, 1910, x, 20.



Prowazek bodies from uncomplicated inclusion cases to the testicles of rabbits, since this organ offers an excellent medium of growth to various highly parasitic organisms which otherwise cannot be easily cultivated.<sup>18</sup> The scrapings of conjunctivæ from 4 different patients were inoculated into the testicles of 8 rabbits; but in spite of the large number of the inclusions contained in the conjunctival scrapings used for this purpose, no success was obtained along this line. The testicles showed within twenty-four hours some induration and edema, but after a few days resumed their normal condition. Tissues removed after twenty-four hours, three days, and six days failed to show any cell inclusions when examined in smears and sections. No hemoglobinophilic organism was found in cultures made from the tissues. This negative finding also tends to strengthen the view that the cell inclusions found in these cases were not the Koch-Weeks bacilli, for if they had been it would have resulted in the production of Koch-Weeks orchitis.

#### CONCLUSIONS.

1. There are cases in which epithelial cell inclusions may alone be present in the conjunctival smears. In such cases no other pathogenic organisms, such as the Koch-Weeks bacillus or the pneumococcus, can be demonstrated in smears or cultures.
2. The conjunctiva can become simultaneously infected with the inclusion bodies and Koch-Weeks bacilli or other organisms.
3. In cases of acute or subacute conjunctival inflammations due to mixed infections the clinical features of each infection may be present. The course of the inflammation is, however, more prolonged.
4. Within recent years, the Koch-Weeks bacillus has only seldom been found in our routine examinations.
5. The epidemic studied was of a severe type.
6. Clinically it is practically impossible to distinguish pneumococcal conjunctivitis from the Koch-Weeks conjunctivitis. Bacteriological examination of smears and cultures is the only means by which the etiological diagnosis can be definitely established.
7. Conjunctivæ of certain species of monkeys are susceptible to

<sup>18</sup> Noguchi, H., *Jour. Exper. Med.*, 1915, xxi, 539.

the von Prowazek inclusion bodies, but not to the hemoglobinophilic bacilli isolated from cases of epidemic conjunctivitis.

8. The injection of conjunctival scrapings containing the von Prowazek cell inclusions into the testicles of rabbits produces no cell inclusions in the latter, while the injection of a pure culture of the hemoglobinophilic bacilli causes an acute inflammation accompanied by numerous clumps of the organisms, simulating the von Prowazek bodies at certain stages of their evolution.

9. There exists an apparent morphological similarity between the degenerated forms of this variety of the hemoglobinophilic bacilli and the cell inclusions, both in cultures and in experimental orchitis in the rabbit. But, as a rule, the elementary bodies of the latter are much smaller and more sharply defined than the smallest granules of the former, while the initial bodies are bigger, more intensely stainable, and less definite in their contour than the hemoglobinophilic bacilli found in the infected conjunctivæ.

#### EXPLANATION OF PLATES.

All the photographs were made from film preparations, except where otherwise stated. They were stained with Giemsa. The enlargement is uniformly 1,000 diameters.

#### PLATE 41.

FIG. 1. This shows a mass of the Koch-Weeks bacilli in a film preparation from an uncomplicated case of Koch-Weeks conjunctivitis. The bacilli belong to the thin type and some scattered examples appear coccoid. By focusing, the mass is seen to be composed of numerous well defined bacilli which cannot be confused with the inclusion granules.

FIG. 2. An epithelial cell from a case of uncomplicated Koch-Weeks conjunctivitis. Around the periphery of the cell and near the nucleus along the lower border numerous bacilli are seen to be attached to, or contained within, the cell body. There is, however, no difficulty in recognizing the bacilli as such in this instance.

FIG. 3. A film preparation from a case of mixed infection of the Koch-Weeks and the inclusion organisms. In the field the epithelial cell is seen to contain a densely stained mass of the initial bodies near the upper right border of the nucleus and numerous Koch-Weeks bacilli to the left, especially where there is a leucocyte.

FIG. 4. This represents a twenty-four hour old pure culture of a thin type of the Koch-Weeks bacilli on blood agar.

FIGS. 5, 6, and 7. The appearance of the same organism as in Fig. 4 after 3 days, 5 days, and 8 days, respectively, on the same medium at 37° C.

FIGS. 8, 9, 10, and 11. These represent the appearance of a medium type strain of the Koch-Weeks bacilli after 24 hours, 3 days, 5 days, and 8 days, respectively, on blood agar at 37° C.

FIGS. 12, 13, 14, and 15. These show the appearance of a strain of round or coccoid type of the Koch-Weeks bacilli in a 24 hour, 3 day, 5 day, and 8 day growth on blood agar, respectively.

FIGS. 16, 17, 18, and 19. These show the appearance of a coarse strain of the Koch-Weeks bacilli in a 24 hour, 3 day, 5 day, and 8 day growth on blood agar, respectively. This strain resembles *B. influenza* more than the others.

FIGS. 20, 21, 22, and 23. These show 4 different strains of the Koch-Weeks bacilli isolated from the mixed infection cases in the present epidemic. They belong to the medium type.

FIGS. 24, 25, and 26. These show 24 hour growths of 3 different strains of *B. influenza* isolated from cases of pneumonia.

FIGS. 27 and 28. These show 24 hour growths of 2 different strains of *B. influenza* isolated from cases of meningitis. Both strains were kept on artificial media for several years and tend to form threads more than they originally did (Wollstein).

#### PLATE 42.

FIGS. 29, 30, 31, 32, and 33. These show the inclusions at various stages of evolution in the film preparations made from a case (Tho.) of inclusion conjunctivitis. This patient had been suffering from the inclusion conjunctivitis when he was superinfected with the Koch-Weeks bacilli, which, in due course, disappeared from his conjunctiva leaving the original condition little affected. These inclusions shown here were found in the conjunctiva long after the Koch-Weeks bacilli had disappeared in smears or in cultures. The minute elementary bodies in Figs. 32 and 33 bear no resemblance to the degenerated forms of the Koch-Weeks bacilli.

FIGS. 34, 35, and 36. These represent the inclusion bodies in an uncomplicated case (Sh.), that is, without any association with the Koch-Weeks bacilli. The character of the initial bodies in Fig. 34 would definitely dispose of any suggestion as to their being the Koch-Weeks bacilli. Fig. 36 shows a still unburst intracellular aggregation of the elementary bodies.

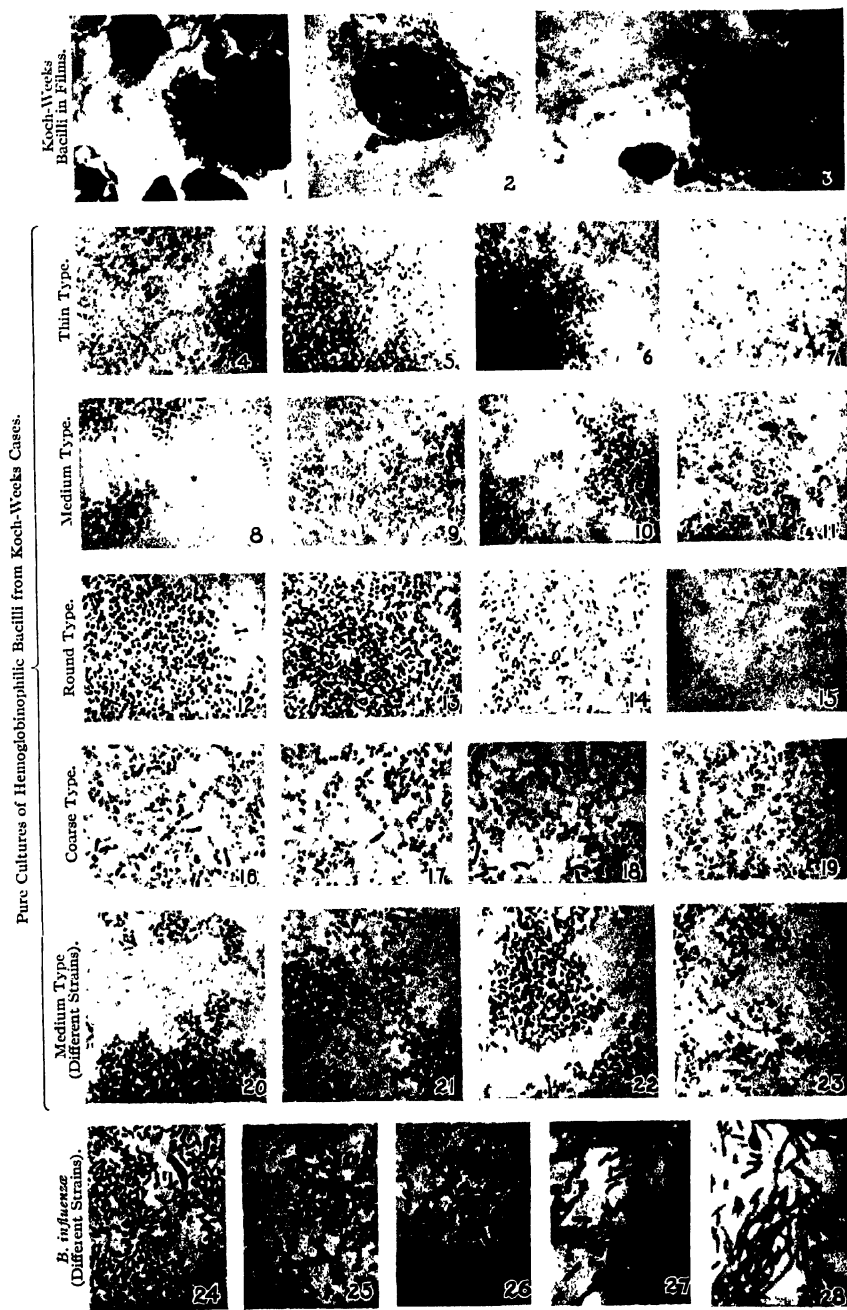
FIGS. 37, 38, and 39. Inclusions from another case (Sat.) of pure inclusion conjunctivitis.

FIGS. 40, 41, and 42. Inclusions from a case (Bol.) of trachoma. The initial bodies as shown in Fig. 40 are exceptionally coarse.

FIGS. 43, 44, and 45. Inclusions from an uncomplicated case (For.) of inclusion conjunctivitis. In Fig. 43 a thick mass of the initial bodies is seen to embrace the left side of the nucleus of a cell on the left, while on the right numerous elementary granules are scattered around the cell below. Figs. 44 and 45 show the steps of evolution of the inclusion bodies.

#### PLATE 43.

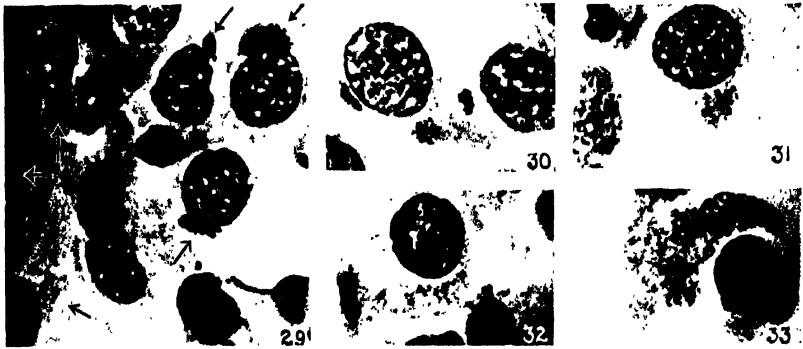
FIGS. 46, 47, 48, 49, 50, and 51. Different stages of the evolution of the inclusion bodies and the conjunctiva of a baboon experimentally infected with the scrapings of the affected conjunctivæ of man. The structure of the initial



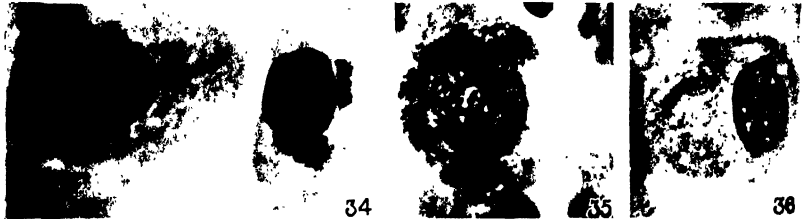


Inclusion Bodies in Film Preparations from Uncomplicated Cases.

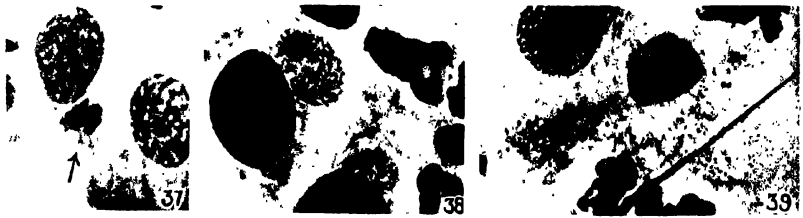
Tho. (29-33).



Sh. (34-36).



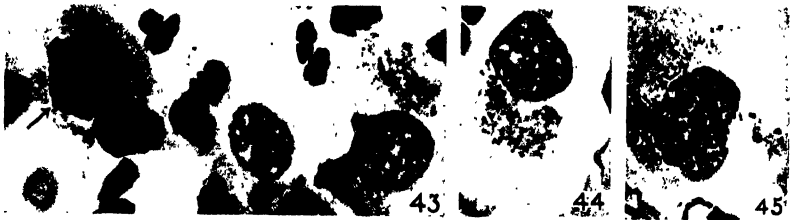
Sit. (37-39).



Bol. (40-42).

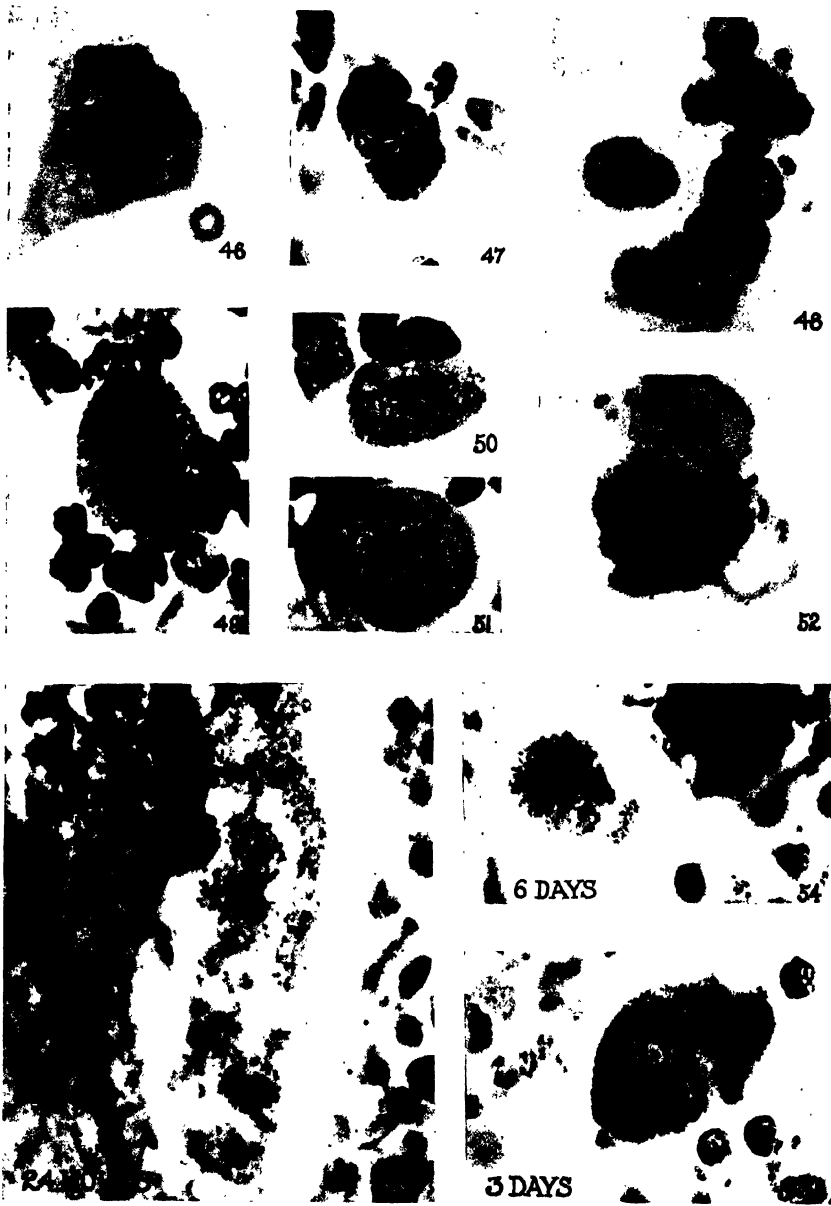


For. (43-45).





Experimental Inclusion Conjunctivitis in Balloon (46-51).



Experimental Koch-Weeks Orchitis in Rabbit (53-55).

(Noguchi and Cohen: Epidemic of Koch-Weeks Bacillus Conjunctivitis.)





bodies in Figs. 46, 47, and 48 is quite peculiar and reveals no definite bacillary outlines. They are deeply stained, almost amorphous, and show indistinct coarse granules within the mass. The elementary bodies are even more minute than those in the human cases, as will be seen in the cell occupying the lower half of Fig. 48. In the same figure one sees an irregularly shaped mass of the initial bodies at the upper corner. In Figs. 49, 50, and 51 are shown the enormously distended cells in which the inclusion granules are rapidly multiplying.

FIG. 52. This shows a dense mass of deeply stained granules in a film preparation from a case of mixed infection. This mass appears to be more of the von Prowazek type of body than of the degenerated Koch-Weeks bacilli.

FIGS. 53, 54, and 55. Sections of the rabbits' testicles inoculated with pure cultures of Koch-Weeks bacilli isolated from the mixed infection cases during the present epidemic. Fig. 53 shows that the organisms assume a coccobacillary form when examined within twenty-four hours after they were injected into the testicle. In photography they appear somewhat like the initial bodies in the inclusion cases, yet in actual examination of the preparations they are not difficult to distinguish as the Koch-Weeks bacilli. They are freely capable of cultivation when a minute portion of the testicle is transferred to the blood agar, while this is not the case with the initial bodies of the inclusion organism. In Figs. 54 and 55 the sections removed after 3 and 6 days, respectively, are shown in which large masses of agglutinated and somewhat ill defined bacilli are found in clear spaces within the lumen of the testicular tubules. They, too, present an appearance not unlike that of the inclusion organism found in experimental conjunctivitis in monkeys, as well as in an epidemic in man. Here, again, a culture on blood agar is quickly differentiated from the other kind, as the Koch-Weeks bacilli at this stage still thrive very well in culture, while the pure inclusion material does not give any growth with the hemoglobinophilic bacilli.

## AN IMMUNOLOGICAL STUDY OF BACILLUS INFLUENZÆ.

By MARTHA WOLLSTEIN, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATE 48.

(Received for publication, June 15, 1915.)

*Bacillus influenza* is a frequent invader of the human body, where it either causes or complicates important pathological processes. Among the pathological conditions which it produces are bronchopneumonia, empyema, and leptomeningitis; and among those in which it accompanies streptococci and pneumococci are the common laryngeal, tracheal, and bronchial affections. Moreover, it is sometimes associated with these cocci in lobular and lobar pneumonia.

According to the location and severity of the infections the influenza bacilli remain confined to the local lesions, or at the same time invade the blood stream. When they are confined to the local lesions in the respiratory organs, the bacilli tend to be of low virulence for animals. When they invade the blood, as they do in leptomeningitis and in some instances of pneumonia, they tend to be of higher virulence. Along with the blood invasion, influenzal suppurative arthritis may occur. These facts suggested the problem as to whether the strains of influenza bacilli isolated from various sources are identical, or whether they may be differentiated into groups on sound biological or serological grounds.

*Sources of the Cultures.*—The strains of *B. influenza* employed in this study came from two main sources; first, the respiratory mucous membrane or the lungs; second, the cerebrospinal fluid. The respiratory strains were usually slightly virulent, the other strains more virulent for laboratory animals. The bacilli isolated from the blood both before and after death were always compared with the respiratory and meningeal cultures obtained during life from the same case. This applies also to the strains isolated from

suppurating joints occurring as a complication of influenzal bacteriemia with meningitis.

### *Criteria of Virulence for Animals.*

The subject of virulence or pathogenicity of *B. influenzae* for animals has been dealt with in previous papers.<sup>1</sup> It will suffice to recapitulate the main facts in this place. The white mouse succumbs to intraperitoneal injections of cultures irrespective of their origin. A peritoneal exudate arises which contains large numbers of the influenza bacilli, as does the heart's blood. Guinea pigs of 200 grams' weight, on the other hand, succumbed to the intraperitoneal injection of one blood agar culture of all the meningeal, and to about one-half of the respiratory, strains tested.

It is by means of the rabbit that the distinction of the virulent or pathogenic strains from those lacking this quality is accomplished. Rabbits of about 1,000 grams' weight are employed. Using one blood agar culture injected intravenously as the test dose, nineteen of the twenty meningeal strains tested caused death within eighteen to thirty hours. The heart's blood in fatal cases contained large numbers of the bacilli. The respiratory strains are for the most part non-pathogenic for rabbits. Among several score of such strains I encountered only six which were virulent for these animals. Four of them came from the lungs and heart's blood and two from the lungs alone (the heart's blood being free) of infants at the Babies' Hospital. In none of the six cases were the leptomeninges involved. In this connection it may be mentioned that Batten<sup>2</sup> described a strain of *B. influenzae* obtained from the meninges which was wholly devoid of pathogenic properties for mice, guinea pigs, and rabbits.

### *Morphology.*

*B. influenzae* is subject to marked variations in morphology. Two main forms are met with, the short and the long. The respiratory strains usually belong to the first class. In films from the bronchial secretion influenza bacilli are short and rather thick, but regular in

<sup>1</sup> Wollstein, M., *Am. Jour. Dis. Child.*, 1911, i, 42; *Jour. Exper. Med.*, 1911, xiv, 73.

<sup>2</sup> Batten, F. E., *Lancet*, 1910, i, 1677.

size. On blood agar plates dew-drop colonies are developed which can be readily differentiated from colonies of all other bacteria by their translucent, colorless appearance. The edges are quite regular, they always remain small, and they never cause hemolysis in the surrounding medium. The bacilli in such colonies are very short and regular, often coccoid in size, with deeply staining poles. No threads are formed (Fig. 4). On blood agar slants the growth is very profuse, but the small translucent colonies do not coalesce. In the condensation water of the tubes threads are formed, but they are never long. The meningeal strains belong to the second class. The bacilli in the cerebrospinal fluid are sometimes remarkably long and thick, and so little do they resemble the usual forms of *B. influenzae* that the diagnosis of influenzal meningitis from films alone becomes difficult (Fig. 1). In other cases short, almost round forms appear in the films so that the presence of a coccus may be suspected (Fig. 2). Grown on moist blood agar plates dew-drop colonies are produced, in which the individuals may be swollen and atypical (Fig. 3). When transplanted to blood agar in which there is less free fluid, the forms become typical. Similarly, an excess of bronchial secretion tends to cause swelling of the bacilli in respiratory strains, but the typical form appears in subcultures. The matter of moisture present affects the morphology of both types. Even so the meningeal strains tend toward larger, more definitely bacillary forms, and incline to the formation of larger threads. When the meningeal strains are recovered from the peritoneal cavity of inoculated animals they are always small and more regular. But the next subculture again shows the larger bacillary forms.

The form tends to be constant for each strain. Thus, a coccoid strain is apt to remain small and regular and to produce only short threads. The bacillary forms, on the other hand, tend to long thread formation. However, after long periods of artificial cultivation (two years or more) both the coccoid and bacillary forms, as they existed, on the one hand, in respiratory and, on the other, in meningeal lesions, grow larger and acquire the power to give rise to long threads (Figs. 5 and 7). If the later generations are examined within the first twenty-four hours of growth some minute bacilli will be detected, indicating the original type to which the strain belongs.

A medium ill suited to growth leads to greater irregularity of form. Thus plain agar tubes upon which a small amount of human blood was placed gave rise upon inoculation of various strains to typical bacilli on the one hand, and, on the other, to long interlacing threads, recalling the leptothrix threads described by Ritchie<sup>3</sup> (Fig. 8). The latter were produced by one meningeal and several respiratory strains. Subcultures in the usual medium from these specimens yielded the typical bacilli. While meningeal and respiratory strains of *B. influenza* differ morphologically they present no points of difference in their method of growth on blood agar plates and slants.

### *Serological Reactions.*

The main purpose of this investigation was, as stated, a minute study of the immunological or serum reactions of a considerable number of strains of *B. influenza*, with the object of determining whether the strains compose one or more groups, irrespective of virulence. For this purpose immune sera were employed, of which the decisive ones were those prepared in the rabbit with selected strains of the cultures.

Monovalent sera were obtained by immunizing rabbits to virulent and non-virulent strains of *B. influenza*. The virulent strains isolated from cases of influenzal meningitis were well borne by the animals in increasing doses over a period of three to five months. The respiratory strains, on the other hand, were badly borne, the rabbits becoming emaciated and dying after a dose which animals inoculated with virulent cultures were well able to bear. From these results it is fair to argue that the non-virulent respiratory strains do not produce immune bodies in sufficient quantities to protect rabbits against repeated and increasing doses of the bacilli.

*Opsonins.*—The opsonic content of the monovalent sera was fairly high, phagocytosis of the organisms being present in dilutions of 1 to 1,000. No specific reaction was obtained, however, the heterologous strains being taken up by the leucocytes in dilutions as high as those in which homologous strains were phagocyted.

*Agglutinins.*—The monovalent rabbit sera did not agglutinate

<sup>3</sup> Ritchie, J., *Jour. Path. and Bacteriol.*, 1910, xiv, 615.

homologous strains *in vitro* in higher dilutions than they agglutinated heterologous strains, and no reaction was obtained above a dilution of 1 to 100.

Bull's<sup>4</sup> experiments have shown that agglutination *in vitro* may give different results from agglutination of the same bacteria *in vivo*. Therefore, his method was used to compare the strains of influenza bacilli. Two young rabbits were inoculated intravenously with a non-virulent respiratory and a virulent meningeal culture, respectively. Blood was taken from the heart at intervals of thirty seconds to eighteen minutes, and slides were prepared. The respiratory culture showed marked clumping within one minute after inoculation, and in five minutes no bacilli could be demonstrated in the blood. The meningeal culture was not agglutinated at all by the blood of the inoculated rabbit, and at the end of eighteen minutes the bacilli were as numerous as they had been half a minute after the injection. The difference in the two results was very striking and clean cut. On killing the animals the bacilli of the respiratory strain were found within leucocytes in the liver, spleen, and lungs, while the virulent (meningeal) bacilli were found only in small numbers in the organs and were extracellular. A third rabbit was inoculated with a meningeal culture which had been isolated two years before, and which had lost its virulence for rabbits. The result was identical with that obtained with the non-virulent respiratory strain, proving again that the difference between the two strains is not absolute but only relative.

*Complement Deviation.*—Antigens were prepared from seven non-virulent respiratory strains and from seven virulent meningitis strains, according to the method used in the research laboratories of the New York Board of Health. I am indebted to Miss Olmstead for a description of this method, which is similar to the one described by Schwartz and McNeil<sup>5</sup> for gonococcus antigens, except that the suspensions of influenza bacilli are kept at 55° C. over night, since they undergo autolysis very slowly. At the end of the period of autolysis, which lasted from fourteen to eighteen hours, a differ-

<sup>4</sup> Bull, C. G., *Jour. Exper. Med.*, 1915, xxii, 484.

<sup>5</sup> Schwartz, H. J., and McNeil, A., *Am. Jour. Med. Sc.*, 1912, cxliv, 815.

ential point between respiratory and meningeal strains of influenza bacilli was noted in the appearance of the fluid. The suspensions of the meningeal cultures were invariably turbid, with a comparatively small precipitate in the tube. The suspension of the respiratory strains, on the other hand, showed a perfectly clear fluid above a large amount of precipitate. In other words, the bodies of the non-pathogenic bacilli underwent less perfect dissolution than did the bodies of the pathogenic strains. The appearances of the precipitates as revealed by stained films under the microscope were quite similar. The bacilli no longer stained deeply and were more or less disintegrated.

All the antigens were tested against two monovalent rabbit sera immune to the meningeal strains, and against two rabbit sera immune to the respiratory strains. The results are given in Tables I to IV.

TABLE I.

*Complement Deviation.**Immune Serum of Respiratory Strains + Antigens of Respiratory Strains.*

Complement. Guinea pig serum 1:40 dilution.	Antigen.	Immune serum (Robinson).	Anti-sheep rabbit serum 1:1,000 dilution.	Sheep corpuscles 1:20 dilution.	Result.	
cc.	cc.	cc.	cc.	cc.		
0.1	1. R.	0.3	0.1	0.25	0.25	No hemolysis.
0.1		0.2	0.1	0.25	0.25	Some hemolysis.
0.1		0.1	0.1	0.25	0.25	Hemolysis.
0.1		0.2	0.05	0.25	0.25	"
0.1	2. S.	0.3	0.1	0.25	0.25	No hemolysis.
0.1		0.2	0.1	0.25	0.25	" "
0.1		0.1	0.1	0.25	0.25	Hemolysis.
0.1		0.2	0.05	0.25	0.25	"
0.1	3. C.	0.3	0.1	0.25	0.25	No hemolysis.
0.1		0.2	0.1	0.25	0.25	" "
0.1		0.1	0.1	0.25	0.25	" "
0.1		0.05	0.1	0.25	0.25	Hemolysis.
0.1		0.2	0.05	0.25	0.25	"
0.1	4. F.	0.3	0.1	0.25	0.25	No hemolysis.
0.1		0.2	0.1	0.25	0.25	Hemolysis.
0.1	5. M.	0.3	0.1	0.25	0.25	No hemolysis.
0.1		0.2	0.1	0.25	0.25	Hemolysis.
0.1	6. M.O.	0.3	0.1	0.25	0.25	No hemolysis.
0.1		0.2	0.1	0.25	0.25	Hemolysis.
0.1	7. L.	0.3	0.1	0.25	0.25	"



TABLE II.

*Complement Deviation.**Immune Sera of Respiratory Strains + Antigens of Meningeal Strains.*

Complement. Guinea pig serum 1:40 dilution.	Antigen.	Immune serum (Robinson).	Anti-sheep rabbit serum 1:1,000 dilution.	Sheep cor- puscles 1:20 dilution.	Result.
cc.	cc.	cc.	cc.	cc.	
0.1	1. D.	0.3	0.25	0.25	No hemolysis.
0.1		0.2	—	—	Hemolysis.
0.1	2. B.H.	0.3	0.1	0.25	No hemolysis.
0.1		0.2	0.1	0.25	" "
0.1		0.1	0.1	0.25	Hemolysis.
0.1		0.2	0.05	0.25	" "
0.1	3. N.Y.H.	0.3	0.1	0.25	No hemolysis.
0.1		0.2	0.1	0.25	Some hemolysis.
0.1		0.1	0.1	0.25	Hemolysis.
0.1		0.2	0.05	0.25	"
0.1	4. Ch.	0.3	0.1	0.25	No hemolysis.
0.1		0.2	0.1	0.25	Some hemolysis.
0.1		0.1	0.1	0.25	Hemolysis.
0.1		0.2	0.05	0.25	"
0.1	5. P.	0.3	0.1	0.25	No hemolysis.
0.1		0.2	0.1	0.25	Hemolysis.
0.1	6. F.A.	0.3	0.2	0.25	"
0.1		0.3	0.1	0.25	"
0.1	7. L.F.	0.3	0.2	0.25	"
0.1		0.3	0.1	0.25	"

Thus it follows that all the sera made by immunizing rabbits with meningeal or with respiratory strains of influenza bacilli contained immune bodies capable of binding complement in the presence of antigens made from both virulent and non-virulent strains. But the sera obtained with the virulent organisms gave binding in higher dilutions than did the sera made from non-virulent bacilli. In other words, the sera obtained by immunizing rabbits with virulent influenza bacilli contained immune bodies capable of uniting with their homologous antigens in comparatively high dilutions, and with heterologous antigens in lower dilutions; while the sera resulting from the inoculation of rabbits with non-virulent influenza bacilli contained less complement binding body for all antigens.

An antigen made from a particular respiratory strain reacted with

TABLE III.  
Complement Deviation.  
*Immune Sera of Meningeal Strains + Antigens of Meningeal Strains.*

Complement. Guinea pig serum 1:40 dilution.	Antigen.	Immune serum.	Anti-sheep rabbit serum 1:1,000 dilution.	Sheep cor- puscles 1:20 dilution.	Result.
cc.	cc.	cc.	cc.	cc.	
0.1	1. B.H.	0.1	0.25	0.25	No hemolysis.
0.1		0.05	0.25	0.25	" "
0.1		0.03	0.25	0.25	" "
0.1		0.01	0.25	0.25	Hemolysis.
0.1	2. Ch.	0.1	0.25	0.25	No hemolysis.
0.1		0.05	0.25	0.25	" "
0.1		0.03	0.25	0.25	Some hemolysis.
0.1		0.01	0.25	0.25	Hemolysis.
0.1	3. N.Y.H.	0.1	0.25	0.25	No hemolysis.
0.1		0.05	0.25	0.25	" "
0.1		0.03	0.25	0.25	" "
0.1		0.01	0.25	0.25	Hemolysis.
0.1	4. F.A.	0.1	0.25	0.25	No hemolysis.
0.1		0.05	0.25	0.25	" "
0.1		0.03	0.25	0.25	Hemolysis.
0.1	5. D.	0.1	0.25	0.25	No hemolysis.
0.1		0.05	0.25	0.25	Hemolysis.
0.1	6. P.	0.1	0.25	0.25	No hemolysis.
0.1		0.05	0.25	0.25	" "
0.1		0.03	0.25	0.25	Hemolysis.
0.1	1. B.H.	2. N.Y.H. 0.1	0.25	0.25	No hemolysis.
0.1		0.05	0.25	0.25	" "
0.1		0.03	0.25	0.25	Some hemolysis.
0.1		0.01	0.25	0.25	Hemolysis.
0.1	2. N.Y.H.	0.1	0.25	0.25	No hemolysis.
0.1		0.05	0.25	0.25	" "
0.1		0.03	0.25	0.25	" "
0.1		0.01	0.25	0.25	Hemolysis.
0.1	3. D.	0.1	0.25	0.25	No hemolysis.
0.1		0.05	0.25	0.25	" "
0.1		0.03	0.25	0.25	Hemolysis.

a heterologous serum (*i. e.*, from a meningeal strain) in lower dilution than with its own serum. However, still another respiratory strain failed to bind at all with any immune serum. Since these results were obtained in repeated tests with antigens made from the two strains and at different times, they are probably not to be regarded as accidental, but as indicating that the respiratory strains differ among themselves in strength of antigenic power. With this conclusion the protection experiments also agree.

TABLE IV.

*Complement Deviation.**Immune Sera of Meningeal Strains + Antigens of Respiratory Strains.*

Complement. Guinea pig serum 1:40 dilution.	Antigen.	Immune serum.	Anti-sheep rabbit serum 1:1,000 dilution.	Sheep cor- puscles 1:20 dilution.	Result.
cc.	cc.	cc.	cc.	cc.	
0.1	1. R.	0.2	0.1	0.25	No hemolysis.
0.1		0.2	0.05	0.25	" "
0.1		0.2	0.03	0.25	" "
0.1		0.2	0.01	0.25	Hemolysis.
0.1	2. S.	0.2	0.1	0.25	No hemolysis.
0.1		0.1	0.1	0.25	Hemolysis.
0.1		0.2	0.05	0.25	" "
0.1		0.3	0.05	0.25	" "
0.1	3. C.	0.3	0.1	0.25	No hemolysis.
0.1		0.2	0.1	0.25	" "
0.1		0.1	0.1	0.25	Hemolysis.
0.1		0.05	0.1	0.25	" "
0.1	4. F.	0.3	0.1	0.25	Some hemolysis.
0.1		0.2	0.1	0.25	Hemolysis.
0.1	1. M.	0.3	0.1	0.25	No hemolysis.
0.1		0.2	0.1	0.25	Hemolysis.
0.1	2. Ma.	0.3	0.1	0.25	No hemolysis.
0.1		0.2	0.1	0.25	Hemolysis.
0.1	3. S.	0.2	0.1	0.25	No hemolysis.
0.1		0.1	0.1	0.25	Some hemolysis.
0.1		0.2	0.05	0.25	Hemolysis.
0.1		0.3	0.05	0.25	" "
0.1	4. C.	0.2	0.1	0.25	No hemolysis.
0.1		0.1	0.1	0.25	" "
0.1		0.05	0.1	0.25	Hemolysis.
0.1	5. L.	0.3	0.1	0.25	" "

While the test of complement deviation brings out a difference between the strains of influenza bacilli isolated from the respiratory tract and those obtained from the meninges, the difference is simply one of degree and not of kind. The sera and antigens made from respiratory strains were both much weaker than were those made from the meningeal strains. This fact is probably explained by the imperfect autolysis of the cultures derived from the respiratory tract and by their inability to produce more than a small amount of protective immune bodies in inoculated rabbits. The antigenic properties of the respiratory cultures are far weaker than are those of the strains isolated from the meninges.

*Protection.*—In order to determine whether the non-virulent respiratory strains which did not kill rabbits elicit the production of immune bodies, the surviving rabbits were reinoculated at various intervals with virulent meningeal strains.

Ten strains of *B. influenzae*, isolated from the respiratory tract, were found to be totally unable to afford any protection to young rabbits against a subsequent inoculation with virulent strains. On the other hand, two respiratory cultures which did not kill rabbits in the ordinary lethal dose (one blood agar slant) of the standard virulent strains did protect the animals from a lethal dose of a virulent culture injected after two to four weeks. As was to be expected, sublethal doses of virulent cultures, whether of meningeal or of respiratory origin, protected rabbits against full doses given twelve days to three weeks later.

It is apparent that the respiratory strains are not identical, but that they differ among themselves in the amount of protective immune bodies they are able to develop in rabbits, just as they differ in the amount of complement binding body they produce.

The following protocols illustrate these results.

### *Protocols.*

*Experiment I.*—Jan. 15. Rabbit, weight 962 gm. Inoculated intravenously with 1 blood agar slant of respiratory strain R of 24 hours' growth suspended in 1 cc. of salt solution.

Jan. 16. Rabbit alive, apparently well.

Jan. 18. Rabbit quite well.

Jan. 30. Rabbit reinoculated intravenously with 1 blood agar slant of meningeal strain N of 24 hours' growth, suspended in 1 cc. of salt solution.

Jan. 31. A. m. Rabbit ill.

P. m., 29 hours after inoculation, rabbit died.

*Experiment II a.*—Jan. 16. Rabbit, weight 975 gm. Inoculated intravenously with 1 blood agar slant of meningeal strain H of 24 hours' growth, in 1 cc. of salt solution.

Jan. 17. Rabbit dead. Profuse growth of *B. influenzae* from heart's blood.

*Experiment II b.*—Jan. 19. Rabbit, weight 975 gm. Inoculated intravenously with ½ blood agar slant of meningeal strain H of 24 hours' growth, suspended in 1 cc. of salt solution.

Jan. 20. Rabbit quite ill.

Jan. 22. Rabbit well.

Feb. 3. Inoculated with one culture of meningeal strain N.

Feb. 5. Rabbit well.

*Experiment III.*—Jan. 19. Rabbit, weight 970 gm. Inoculated intravenously with 1 blood agar slant of respiratory strain C of 24 hours' growth, suspended in 1 cc. of salt solution.

Jan. 20. Rabbit well.

Feb. 10. Inoculated intravenously with 1 culture of meningeal strain N in 1 cc. of salt solution.

Feb. 15. Rabbit well.

#### SUMMARY.

Influenza bacilli isolated from various pathological processes in man differ widely in pathogenic power for animals, especially rabbits. While the cultures derived from the leptomeninges and blood, and rarely from the pneumonic lung are pathogenic, those generally derived from the respiratory tract exhibit little or no virulence for rabbits.

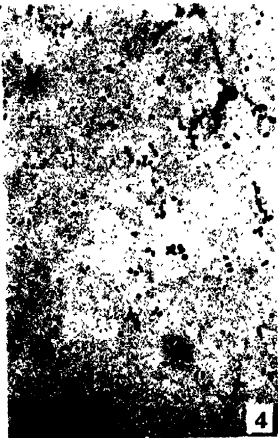
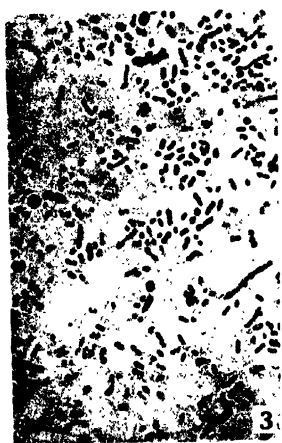
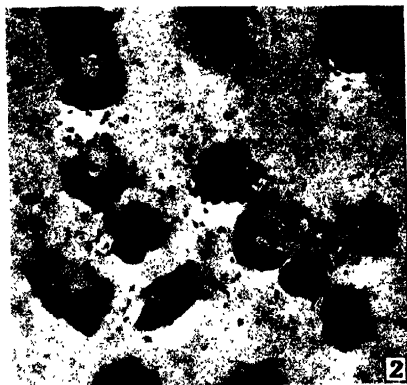
The two types of cultures as indicated by virulence for animals do not differ in kind, but only in degree, in relation to the serological tests of agglutination, complement deviation, and opsonification.

The two types of cultures do, however, differ with respect to their ability to undergo autolysis. While the virulent cultures autolyze almost completely, yielding a turbid supernatant fluid and little sediment, the non-virulent cultures give rise to an abundant sediment and a clear supernatant fluid.

The non-virulent cultures incite far less antibody production in rabbits. Hence, rabbits inoculated with non-virulent strains yield sera possessing low antibody content. Conversely, rabbits inoculated with virulent strains yield sera possessing a higher content of antibody.

In keeping with and possibly because of the low antibody content of the sera of rabbits inoculated with the non-pathogenic strains, the rabbits so treated are not, as a rule, protected against subsequent inoculation with virulent strains.

Influenza bacilli therefore vary in pathogenic effect both for man and animals, but they are not distinguishable by means of serological reactions into different types. Apparently all influenza bacilli belong to one class or race irrespective of origin or virulence.





EXPLANATION OF PLATE 48.

FIG. 1. Large forms of *B. influenzae* in a film from cerebrospinal fluid in a case of seropurulent leptomeningitis.

FIG. 2. Small forms of *B. influenzae* in a film from cerebrospinal fluid in a case of seropurulent leptomeningitis. Some phagocytosis.

FIG. 3. Impression from a colony of *B. influenzae* on a moist blood agar plate. Note long and swollen forms, also polar staining. Meningeal strain.

FIG. 4. Characteristic small type culture of *B. influenzae*; 20 hours' growth. Respiratory strain.

FIG. 5. Large type culture of *B. influenzae* after 2 years of artificial cultivation.

FIG. 6. Same strain as Fig. 5 from peritoneal cavity of guinea pig 24 hours after inoculation.

FIG. 7. Small type of *B. influenzae* after 2 years of artificial cultivation.

FIG. 8. Culture of *B. influenzae* on blood smeared agar, showing interlacing threads.



## THE MECHANISM OF THE CURATIVE ACTION OF ANTIPNEUMOCOCCUS SERUM.

By CARROLL G. BULL, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 49 TO 52.

(Received for publication, June 1, 1915.)

In a previous paper<sup>1</sup> I reported on the course of the septicemia in the rabbit which follows an intravenous injection of pneumococci. In brief it is as follows: By making blood cultures at frequent intervals it was found that the course was determined largely by the number and virulence of the bacteria injected. The injection of a relatively small number of pneumococci of low virulence is followed in a few hours by a sterile condition of the blood, and the bacteria do not reappear in the circulation. When large numbers of similar pneumococci are injected or when a small number of a virulent strain is given, the initial decrease in the number of bacteria in the blood takes place less rapidly and completely and, after an interval of five or six hours, an increase occurs. When, however, large numbers of virulent pneumococci are injected, a slight initial fall occurs and death results in a few hours from severe bacteremia.

These results, it was noted at the time, could be completely changed if a small quantity of active antipneumococcus serum was injected. Under these conditions the bacteria even when highly virulent were abruptly swept from the blood. No explanation of the phenomenon was attempted at the time. It is well known that the immune serum is devoid of bactericidal properties *in vitro*. On the other hand, bacteriotropic substances are demonstrable both *in vitro* and *in vivo* and Neufeld and others believe that a relation exists between bacteriotropic value and protective action.<sup>2</sup> The

<sup>1</sup> Bull, C. G., *Jour. Exper. Med.*, 1914, xx, 237.

<sup>2</sup> For previous work and literature on these points the following references are given: Neufeld, F., and Rimpau, W., *Ztschr. f. Hyg. u. Infektionskrankh.*, 1905, li, 283. Boehncke, K. E., and Mouriz-Riesgo, J., *Ztschr. f. Hyg. u. Infektionskrankh.*, 1915, lxxix, 355. Cole, R., *Arch. Int. Med.*, 1914, xiv, 56.

question therefore arises as to what the abrupt disappearance of the pneumococci from the circulation is due and whether it is the result of the action of one or more of the antibodies contained in the blood and the immune serum, and which of them plays the essential part.

#### EXPERIMENTAL.

The investigation of the pneumococcus was rendered far easier than it otherwise would have been by the fact that the Hospital of The Rockefeller Institute kindly supplied several types<sup>3</sup> of the pneumococcus with their corresponding immune horse serum. Although the majority of the experiments were made with a virulent pneumococcus of Type I, Types II and IV were also employed to supplement and extend the study. Since the investigation of the blood of the rabbits, employed for the tests, during life proved an essential part of the experiments, a brief description of the procedures will be introduced here. They will not be repeated in connection with the illustrative experiments given.

*Blood Cultures.*—The number of bacteria in the blood stream was determined by taking a small quantity of blood from the heart with a graduated pipette to which a needle was attached by means of a thick-walled rubber tube. A measured quantity of the blood was immediately diluted with a large quantity of sterile physiological salt solution and portions of the dilutions were added to Petri dishes containing sterile defibrinated rabbit blood. The colonies were counted after twenty-four hours' incubation. The plating was done in this manner throughout the work.

*Microscopic Examination of the Blood.*—For microscopic examination the blood was obtained as described above and heavy films were made on slides. The films were allowed to dry in the air, fixed in methyl alcohol, and stained with a dilute solution of Manson's stain. In this way every bacterium is rendered visible, independently of the thickness of the smear.

*Microscopic Examination of the Tissues.*—Tissues were prepared for microscopical study by crushing and sectioning. For the crushed preparations, the fresh tissues were finely crushed in tissue crushers, portions of the pulp spread on slides, fixed in a flame,

<sup>3</sup> Dochez, A. R., and Gillespie, L. J., *Jour. Am. Med. Assn.*, 1913, lxi, 727.

and stained by Gram's method. Sections were stained by Gram's method, eosin and methylene blue, and hematoxylin and eosin.

*Immune Serum in Pneumococcus Septicemia in Rabbits.*—It has been stated that the immediate effect of the injection of an active immune serum into the blood of rabbits in which many pneumococci are present is to bring about an abrupt disappearance of the bacteria from the circulation, although they may later return. This effect is illustrated by Experiment I in which a bacteremia is produced by the injection of a culture of virulent pneumococcus.

*Experiment I.*—4 rabbits received each in the ear vein an intravenous injection of 0.4 cc. per kilo of an 18 hour bouillon culture of a Type I pneumococcus. 2 of the 4 animals received an intravenous injection 1 minute later of the homologous immune serum in the opposite ear vein, while the remaining 2 were untreated. Blood cultures, by the method described, were made from all the animals. The result is summarized in Table I.

TABLE I.

Rabbit No.	Weight.	Immune serum per kilo.	Colonies of bacteria 1 minute after serum injection.	Colonies of bacteria 15 minutes after serum injection.	Remarks.
I	gm. 1,550	cc. 0.2	1,320,000	0	Bacteremia after 12 hrs.; death in 36 hrs.
II	1,650	0.5	1,250,000	0	Bacteremia; death in 48 hrs.
III	1,850	0	1,260,000	1,100,000	Bacteria did not leave blood; death within 24 hrs.
IV	1,575	0	1,330,000	1,275,000	Same as Rabbit III.

As Experiment I shows, the immune serum brings about a rapid disappearance of the bacteria from the blood. Other experiments directed to the answer of the question indicated that the time required for the blood to be rendered sterile depends, first, on the number of the bacteria injected, and, second and especially, on the amount of serum given. The phenomenon of abrupt removal seems to be independent of the virulence of the pneumococci, although the end result is determined by the virulence. It can be predicted that had the pneumococci been of lower virulence Rabbits I and II would have recovered and the bacteria would not have reappeared in the blood at all.

*Mechanism of the Removal Process.*

The immediate effect, therefore, of the immune serum is to cause the pneumococci to be rapidly removed from the blood stream. This action is exercised independently of the end result; namely, whether the bacteria are to be permanently suppressed, or whether they reappear later and cause the death of the animals. The question which presents itself for solution is the manner in which the removal is accomplished.

It is known that an immune serum even in the fresh state is devoid of bactericidal action on pneumococci *in vitro*. It was desirable to ascertain the effect of the whole blood under the same conditions. Tests were made by adding to fresh rabbit's blood, either hirudinized or defibrinated, varying quantities of immune serum and a convenient number of pneumococci and then plating the mixture at stated periods during twenty-four hours. It sufficed to add two drops of a bouillon culture, twenty-four hours old, to each cc. of the blood and immune serum mixture.

The effect of the immune serum was to cause great reduction in the number of colonies developing on the plates made from the second to the twelfth hour. The later plates showed increasing numbers of colonies and those made at the expiration of twenty-four hours corresponded with the control plates. The smallest number of colonies on a plate was ten to twenty; sterility was never achieved.

In following with the microscope the changes taking place in the test-tubes it was found that what appeared to be destruction of the pneumococci was actually an agglutination of the bacteria through which the number of colonies was reduced. Definite clumps were discovered in tubes in which the proportion of immune serum was 1 to 500. This fact is noteworthy since the macroscopic agglutination titer of the serum was 1 to 50.

*Agglutination and Phagocytosis in Vivo.*

It now became desirable to investigate the same points under the conditions of the living body. Experiments 2 and 3 were performed for that purpose.

*Experiment 2.*—A rabbit having been inoculated by intravenous injection with a small quantity of a bouillon culture of a virulent pneumococcus was permitted to develop a severe bacteremia which required about 10 hours. At that time 1 cc. of immune serum was injected into the ear vein, after which specimens of blood were taken from the heart at intervals of from 30 seconds to 5 minutes. Film preparations were prepared with each specimen, fixed in methyl alcohol, and stained with Manson's stain.

The specimen taken before the serum was injected showed numerous diplococci distributed evenly throughout the blood. The specimen taken 1 minute after the serum was given revealed large clumps of diplococci (Figs. 1 and 2). The next specimen removed showed progressively fewer clumps until the 5 minute specimen was reached, when no diplococci whatever were found.

At the expiration of 30 minutes after the injection of the immune serum the rabbit was killed. The lungs, liver, spleen, and kidney were put through the crusher, and film preparations were made from each. Clumps of diplococci were found in the lungs, liver, and spleen (Figs. 3, 4, and 5), while very few clumps were present in the kidney. The masses of diplococci were sometimes free, but usually they were contained within phagocytic cells and chiefly within polymorphonuclear leucocytes, especially in the lung, liver, and spleen (Figs. 6, 7, 8, and 9). Sections of the organs were also prepared, but they were less informing than the crushed tissues. The sections exhibited, however, groups of leucocytes in the capillaries and other small vessels of the lungs, the sinusoids of the liver, and the blood spaces of the spleen, of which a part enclosed clumps of diplococci (Figs. 12, 13, 14, 15).

*Experiment 3.*—In this animal the equivalent of a developed bacteremia was induced by injecting into an ear vein the virulent pneumococci derived by centrifugalization from 50 cc. of a twenty-four hour old bouillon culture. After 2 minutes a film of the blood taken from the heart was made and then 1 cc. of immune serum injected. Subsequently film preparations of the heart's blood were prepared at periods of from 30 seconds to 5 minutes.

The results were identical with those described in Experiment 2. The blood film taken before the serum was injected showed many diplococci but no clumps; 30 seconds after the serum injection large clumps were present; the crushed organs showed clumped diplococci within phagocytes.

The two experiments were repeated many times and with unvarying appearances. They were also modified in such a manner as to permit the treated rabbits to survive 1, 2, 3, and 4 hours after the injection of the immune serum. A marked difference was observed in the number and condition of the clumps of bacteria according to the period of survival. The greatest number of free clumps of diplococci were present in the animals killed at the 30 minute period, and the greatest number of phagocytes carrying the clumps were found in the animals killed at one and at two hours (Fig. 10). The preparations of animals killed at two and one-half hours show-

ing the phagocytes containing diplococci are fewer and the form of the bacteria is irregular and the staining faint and poor. Later preparations show very few phagocytes enclosing disintegrated bacteria (Fig. 11). The preparations at four hours contained a small number of free, single diplococci which were the last bacteria to disappear.

So far as the blood phenomena are concerned—the abrupt disappearance of the cocci from the circulating blood, agglutination, and phagocytosis—active and passive immunity are similar. The actively immune rabbits probably possess a tissue immunity which passive immunity does not confer. This point is under investigation.

#### DISCUSSION.

It is now possible to define clearly the effects exerted by an anti-pneumococcus serum when it brings about an amelioration of the infective pneumococcus process in rabbits, at least. It may be well to state at the outset that the effects were, in our experiments, limited to the interaction of the specific type or group of pneumococcus and homologous serum. No crossed or heterologous action was ever observed. Although a few typical or illustrative experiments in which Type I pneumococcus and serum were employed are given, similar experiments were made and identical results obtained with pneumococci and serum of Types II and IV.

Setting out with the condition of pneumococcus bacteremia of rabbits, developing on the one hand after a small inoculation of virulent pneumococci and directly produced on the other by an injection of massive quantities of cultures, the first effect of an injection of immune serum is to cause an almost instantaneous agglutination of the diplococci in the blood and the immediate removal of the clump formed by the spleen, liver, lungs, and, to a smaller extent, the kidneys. Probably still other organs participate in this process.

This agglutination and removal by the organs of the pneumococci is quickly followed by a process of active phagocytosis, chiefly through the medium of the polymorphonuclear leucocytes, in the course of which the bacteria are taken up by the cells in enormous

numbers. A single leucocyte may contain from fifty to one hundred diplococci. The act of phagocytosis is directed, apparently, exclusively or almost so against the clumps, while the few single diplococci remain free within the vessels. The act of phagocytosis follows quickly upon the agglutination and removal and, it would appear, never takes place in the blood stream itself; for in the study of hundreds of blood films made from the heart only one leucocyte containing diplococci was encountered. The process extends, however, over several hours and consists of two distinct phases: one the mere englobing of the masses of diplococci, and the other their digestion or disintegration. For the unagglutinated diplococci which remain free retain form and staining powers very much longer than those contained within phagocytes. The diplococci within the phagocytes show at first normal morphology and staining (Figs. 6, 7, 8, and 9), while, later, bizarre forms and imperfect staining are met with (Fig. 11). After a few hours only very few diplococci and those disintegrating are still visible in the phagocytes. Cells other than polymorphonuclear leucocytes play a minor part in the phagocytic process.

The intimate interaction of agglutination and protection which the experiments indicate is emphasized in other ways. I have found that the smallest quantity of immune serum which influences a pneumococcic infection in the rabbit by diminishing the number of diplococci in the circulating blood or by prolonging the life of the animal brings about clump formation *in vivo*; and Avery has noted that in the fractionated and purified antipneumococcic serum the protective bodies are always accompanied by agglutinating substances.

But in order that the agglutinated diplococci may be rendered harmless it is not enough that they shall be removed from the blood by the organs; they must be further withdrawn, apparently, into the phagocytes where they are quickly disintegrated. As the experiments *in vitro* with the whole blood and immune serum showed, the clumped diplococci are capable of further multiplication. Hence, along with the removal of the clumps of diplococci by the organs it is necessary that an accumulation of polymorphonuclear leucocytes in the same organs be brought about simultaneously.

The fact is well known that the intravenous injection of foreign protein causes a leucopenia. I found that the intravenous injection in rabbits of one cc. of antipneumococcus horse serum led, within five minutes, to a reduction of the number of circulating leucocytes by one-half. The low number persisted from three to four hours, after which it rose usually somewhat above the normal. This reduction and rise in number was not participated in by all the white cells but chiefly by the polymorphonuclear leucocytes. Normal horse serum produced similar effects. The injection of bouillon cultures of pneumococci causes a greater fall still in the number of circulating leucocytes and, according to the final result in death or recovery, there was continued depression or later rise.

The manner in which the leucocytes react to the foreign protein as contained in the horse serum provides a mechanism for bringing large numbers of polymorphonuclear leucocytes into close proximity with the agglutinated diplococci within the organs. That the leucocytes are not destroyed but merely accumulate in the organ in certain forms of leucopenia has been shown by Goldschieder and Jacob. On the other hand, an homologous immune serum,—for example antipneumococcus serum prepared in the rabbit,—would also bring about agglutination of the diplococci but whether it would equally produce leucopenia has still to be determined. Some other means of attracting leucocytes from the blood and the bone marrow to the capillaries of the organs may be necessary and this means may well have to do with the presence of the clumps of diplococci themselves. But this constitutes a point of further study.

#### SUMMARY.

An active antipneumococcus serum causes agglutination of pneumococci *in vitro* and *in vivo*.

The antiserum acts in far greater dilution in causing agglutination as determined by the microscopic than by the macroscopic test.

The antiserum when injected into the circulation of rabbits suffering from pneumococcus bacteremia causes a rapid disappearance of the diplococci from the blood. This disappearance is brought about by instantaneous clumping of the diplococci *in vivo* and the removal of the clumps by the liver, spleen, lungs, and possibly other organs.



The same means which cause the clumping and removal from the blood of the diplococci and their accumulation in the organs cause also leucopenia with accumulation of polymorphonuclear leucocytes in the same organs. The leucocytes act as phagocytes and ingest the clumped diplococci which undergo rapid dissolution within the cells.

The diplococci which fail of agglutination tend not to be phagocyted and persist longer in viable form than the ingested clumps.

The protection afforded by the immune serum is specific for the type organism and is determined by the presence of agglutinins which prepare the pneumococci for removal from the blood and phagocytosis on a large scale.

Normal rabbit blood is devoid of agglutinating and of protective effect against virulent pneumococci.

Agglutination is not merely an incidental phenomenon, but constitutes an essential process in association with phagocytosis in the protection of the rabbit against pneumococcus infection.

#### EXPLANATION OF PLATES.

##### PLATES 49 AND 50.

FIG. 1. Clump of pneumococci in heart's blood of a rabbit having a pneumococcic septicemia; specimen was taken 1 minute after 2 cc. of immune serum were given. Manson's stain.

FIG. 2. Clump of pneumococci in heart's blood of a rabbit 1 minute after 0.1 cc. of serum was given.

FIGS. 3, 4, and 5. Clumps of pneumococci in the liver, lungs, and spleen, respectively, of a rabbit 30 minutes after serum had been administered. Gram's stain.

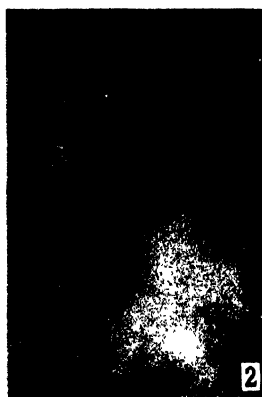
FIGS. 6, 7, 8, and 9. Polymorphonuclear leucocytes containing clumps of pneumococci from the liver, spleen, lungs, and kidney, respectively, of a rabbit having a pneumococcic septicemia. The tissues were removed 1 hour after an injection of immune serum. Gram's stain.

FIG. 10. Crushed preparation from the spleen of a rabbit having a pneumococcic septicemia. 2 leucocytes are present which contain clumps of pneumococci. The tissue was removed 1½ hours after an injection of immune serum had been given. Gram's stain.

FIG. 11. Same as Fig. 10. Tissue removed 2 hours after the serum was given. 1 leucocyte contains a clump of pneumococci. The bacteria stain poorly.

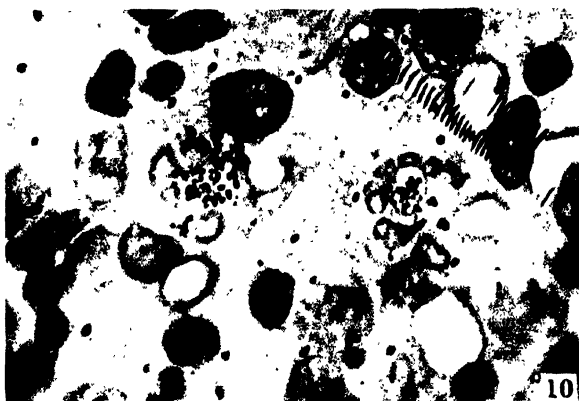
##### PLATES 51 AND 52.

FIGS. 12, 13, 14, and 15. Sections of tissue from liver, spleen, and lungs, respectively, of a rabbit 2 hours after an intravenous injection of 1 cc. of a bouillon culture of pneumococci. All sections show accumulated polymorphonuclear leucocytes. Stained with hematoxylin and eosin.



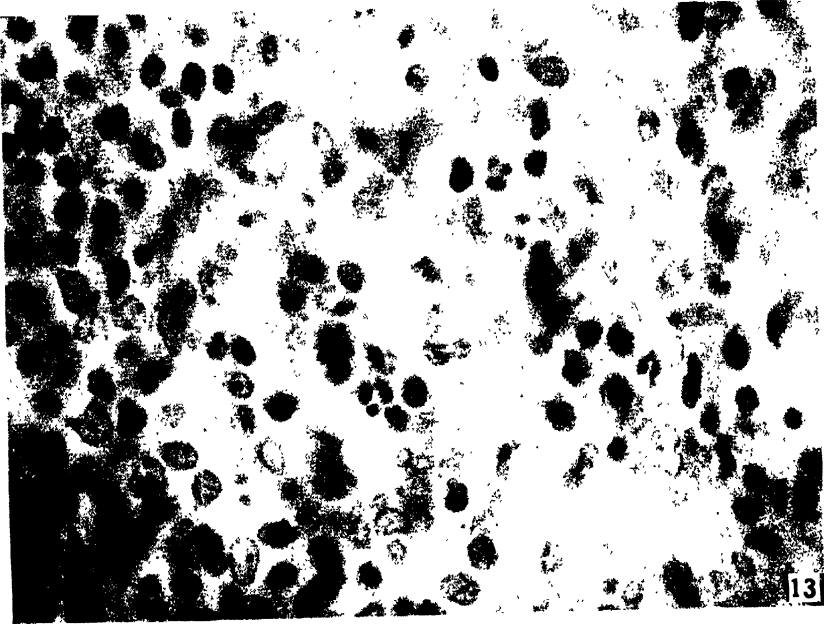
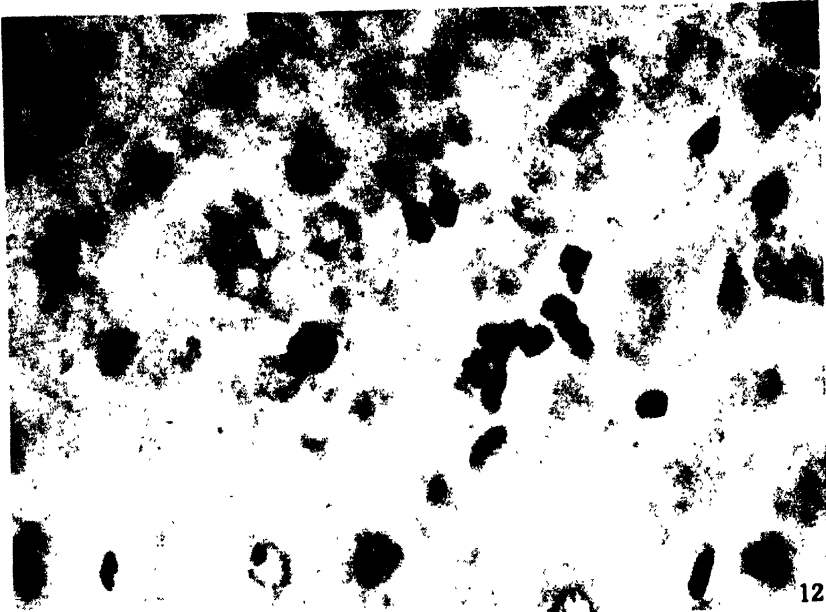
(Bull: Curative Action of Antipneumococcus Serum.)





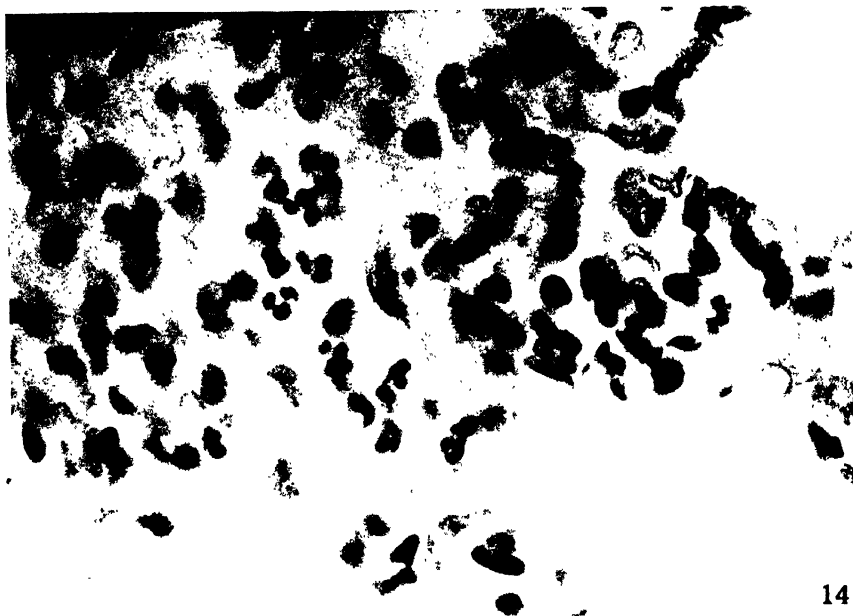
(Bull: Curative Action of Antipneumococcus Serum.)



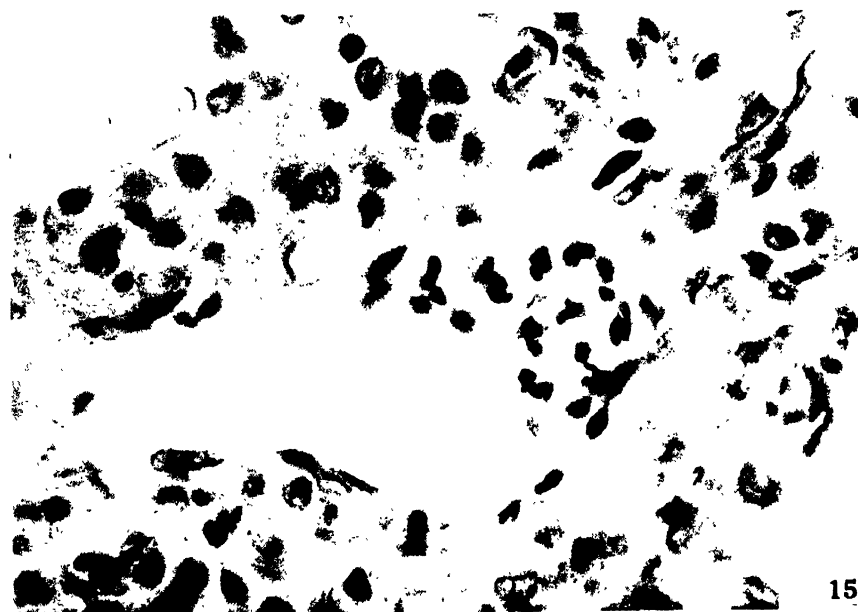


(Bull: Curative Action of Antipneumococcus Serum.)





14



15

(Bull: Curative Action of Antipneumococcus Serum.)





## A METHOD OF SERUM TREATMENT OF PNEUMO- COCCIC SEPTICEMIA IN RABBITS.

By CARROLL G. BULL, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATE 53.

(Received for publication, June 1, 1915.)

In a separate paper<sup>1</sup> I have reported experiments which indicate that the removal of pneumococci from the circulation in rabbits and their destruction are determined by three main factors: (1) the agglutination of the bacteria in the blood stream; (2) the assembling of the clumps in the internal organs; and (3) the inclusion of the masses by and digestion within polymorphonuclear phagocytes. In order that these several processes may occur it has been found necessary, in the case of virulent pneumococci, to employ a suitable immune serum.

In this paper I propose to describe still other experiments which bear upon the above conception of the manner of interaction of immune serum and the animal body. It has hitherto been ascertained that the activity of an immune antipneumococcic serum is quite strictly limited. Beyond a given infecting dose of the pneumococci any practicable quantity of immune serum ceases to be protective. Indeed, as the dose of serum is increased, especially when it comes from a foreign species of animal, a point is reached at which the serum becomes deleterious rather than helpful. The precise conditions upon which this change of action depends are not known; it is surmised that a heterologous serum, in small experimental animals especially, may exert a toxic action which becomes noticeable in effect when the dose is relatively large. In any case a striking disparity exists between the unlimited neutralizing action of the antitoxic sera, diphtheria and tetanus sera, for instance, and the strictly limited anti-infectious action of the antibacterial sera, among which the antipneumococcus serum is to be classed.

<sup>1</sup> Bull, C. G., *Jour. Exper. Med.*, 1915, xxii, 457.

Although the literature on the anti-infectious power of antipneumococcic serum is voluminous, the chief recent important contributions to the subject are those of Neufeld and Haendel<sup>2</sup> and of Cole.<sup>3</sup> The former investigators established the fact of the existence of specific types of pneumococci which are subject to influence only by the corresponding immune sera; and the latter, together with his coworkers, besides extending the knowledge of the types of pneumococci and their specific antisera, observed also that up to a certain degree of infection the protective dose of serum is parallel to the quantity of culture inoculated, after which the quantity of serum required to save life is proportionally large, and finally, a degree of infection may be reached against which no amount of serum will protect. For example, 0.2 cc. of serum of Type I will regularly protect a mouse against 0.1 cc. of a Type I culture, no matter how high its virulence. That is the largest dose of culture, however, against which the immune serum will protect, no matter how much serum is employed. 1 or even 2 cc. are no more effective than 0.2 cc. (Cole).

In view of these data, it has been long believed that the animal body supplied a necessary substance which cooperated with the immune serum in overcoming bacterial infections. The nature of this substance has been conjectured merely; there exists no actual knowledge of its nature. The experiments reported in the previous paper referred to<sup>4</sup> seem to indicate quite definitely that the part which the rabbit's body itself plays in overcoming pneumococcus and some other bacterial infections is supplied by the phagocytes. The experiments to be related here bear on this conception of the protective mechanism.

#### EXPERIMENTAL.

As has just been stated, experiments seemed to indicate that the phagocytic cells function as the chief defensive agents against infection. If this is true, then the limit of the effective action of an immune serum may possibly be extended by giving the phagocytes assistance and providing them sufficient time in which to do their work. We observed that when a rabbit suffering from septicemia is given a

<sup>2</sup> Neufeld, F., and Haendel, *Ztschr. f. Immunitätsforsch., Orig.*, 1900, iii, 159.

<sup>3</sup> Cole, R. I., *N. Y. Med. Jour.*, 1915, ci, 1, 59.

<sup>4</sup> Bull, *loc. cit.*

large injection of immune serum intravenously, the bacteria are agglutinated into massive clumps (Figs. 1 and 2) most of which accumulate in the lungs where they come imperfectly under the influence of the phagocytes and may even, through extensive capillary obstruction, interfere with the circulation. The experiments to follow were performed on rabbits with a strain of pneumococcus of Type I maintained at high virulence by continuous passage through these animals. The corresponding immune serum was prepared in the horse and kindly supplied by the Hospital of The Rockefeller Institute.

The virulence of the culture was not accurately titrated. It was such, however, that one drop of the blood of a rabbit succumbing to infection sufficed to kill in twelve hours a rabbit weighing two kilos. The pneumococci were grown in beef infusion broth and were eighteen to twenty-four hours old when used. When large quantities were to be injected the bacteria were thrown out of the culture in the centrifuge, the greater part of the fluid was poured off, and the remainder suspended for injection. The bacteria were not washed. The injections both of bacteria and serum were made intravenously.

*Experiment 1. Effect of Large Serum Injection in Rabbits Suffering from Severe Pneumococcus Septicemia.*

*Rabbit A.*—Weight 1,800 gm. 0.1 cc. of culture given. 8 hours later the blood contained large numbers of pneumococci. 4 cc. of immune serum injected. The animal died within 1 minute of respiratory failure.

*Autopsy.*—Large clumps of pneumococci in the heart's blood; the lungs were distended, and many clumps of bacteria were in the vessels; clumps of bacteria also in the vessels of the choroid plexus (Figs. 1 and 2).

*Rabbit B.*—Weight 1,600 gm. 0.1 cc. of heart's blood of a rabbit just dead of pneumococcus septicemia given. 6 hours later the blood revealed the existence of a severe septicemia with the diplococci uniformly scattered throughout. 2 cc. of immune serum were injected and films from the heart's blood were made every minute for 3 minutes.

The immediate effect of the serum was to produce collapse of the animal attended by labored respiration and urination. The 1 minute film showed many large clumps of bacteria, the 2 minute fewer clumps, and the 3 minute few small clumps. The rabbit survived, temporarily recovered, but died 6 hours later. The blood and organs contained many pneumococci.

*Rabbit C.*—Weight 2,200 gm. The sediment from 50 cc. of a bouillon culture injected. 2 minutes later 10 cc. of immune serum given in opposite ear vein. The blood was quickly cleared of the pneumococci. Death occurred after 12 hours.

*Autopsy.*—Lungs edematous, emphysematous, and hemorrhagic. Small hemorrhages in surface of the kidneys and peritoneal surface of the intestines. The blood, lungs, spleen, and liver contained very great numbers of pneumococci.

*Rabbit D.*—Weight 2,300 gm. The sediment from 50 cc. of bouillon culture injected. No further treatment. Death in 4½ hours. The blood and organs contained very many pneumococci.

The experiments indicate that when the blood of the rabbit contains a very large number of pneumococci the intravenous injection of a large amount of immune serum causes certain definite effects. In the first place, the effect may be to cause almost immediate death. This accident results from the rapid agglutination in large clumps of the pneumococci in the circulation and the massing of the clumps in the lungs and brain where, acting as emboli, they produce respiratory failure and death. In the second place, the blood may be temporarily cleared of the bacteria and life be prolonged. Finally, however, the life of the animal is not spared, as the bacteria reinvade the blood and cause death.

From the foregoing it appears that the formation of large clumps is a disadvantage to the animal, since they tend to be held back chiefly in the lungs, the circulation of which they obstruct; and, besides, the large bacterial masses are not readily phagocyted.

These disadvantages can be avoided by the injection of small quantities of immune serum which produce clumps containing twenty to thirty pneumococci and to their quite regular distribution in the lungs, liver, and spleen, where they come under the influence of phagocytes under favorable conditions. Once removed from the circulation and lodged in the organs, the effect of a larger quantity of serum was studied.

*Experiment 2. Effect of First Small and Later Larger Doses of Immune Serum in Pneumococcus Septicemia.*

*Rabbit A.*—Weight 1,800 gm. The sediment from 75 cc. of a bouillon culture given. Immediately 0.5 cc. of immune serum injected into opposite ear vein. At the examination 25 minutes later there were no bacteria in the blood. 5 cc. of immune serum injected. Animal showed no symptoms for 2 hours, after which symptoms appeared, and death occurred at end of 5 hours.

*Autopsy.*—The lungs were edematous and hemorrhagic. Hemorrhages in surface of kidneys and peritoneal coat of intestine. The blood was free of diplococci and very few were found in the spleen, lungs, and liver. Almost all had been destroyed.

*Rabbit B.*—Sediment from 75 cc. bouillon culture administered. No treatment. Died in 6 hours. Blood and organs teeming with bacteria.

The experiment recorded under Rabbit A was repeated several times but without changing the end result. In spite of the practical destruction of the pneumococci, death quickly resulted. The appearance of the organs, and especially the hemorrhages, were taken to indicate a severe grade of intoxication,<sup>5</sup> and this effect was provisionally related to the large second dose of serum leading to too rapid disintegration of the diplococci. Hence it was decided to bring about a more gradual disintegration of the bacteria if possible.

*Experiment 3. Effect of Repeated Small Doses of Immune Serum in Pneumococcic Septicemia.*

*Rabbit C.*—Weight 2,000 gm. The sediment from 150 cc. of a bouillon culture given. Two minutes later 0.5 cc. of immune serum injected. At the end of 30 minutes the bacteria had left the blood and 1 cc. of the serum was administered. From now on 1 cc. of the serum was administered every 2d hour. Although symptoms appeared at the end of the first 2 hours they disappeared and the rabbit was in good condition until the 22d hour, when restlessness and rapid respiration followed by rigidity of hind leg developed. Later opisthotonos supervened. A lumbar puncture yielded cerebrospinal fluid containing many diplococci. Death occurred in the 28th hour.

*Autopsy.*—Blood, spleen, liver, and lungs were free of bacteria. The meninges were inflamed and contained very large numbers of pneumococci. Death obviously resulted from meningitis.

*Rabbit D.*—The sediment from 100 cc. of bouillon culture was given. No treatment. Died in 4 hours. The blood and organs contained very large numbers of diplococci.

The experiment described in Rabbit C was made several times but always with the same final result. The success of the experiment was frustrated by the intervention of the meningitis. The possibility is not excluded that this complication may itself be prevented by bringing a proper dose of the immune serum into the meninges which are not reached from the circulating blood.<sup>6</sup> The effect of reducing the infecting dose of bacteria was next tried. The sediments from 75 cc. and from 50 cc. of bouillon culture only delayed the onset of meningitis. The sediments from 35 cc. of culture gave partially successful results.

<sup>5</sup> Sprunt, T. P., and Luetscher, J. A., *Jour. Exper. Med.*, 1912, xvi, 443.

<sup>6</sup> Flexner, S., *Jour. Am. Med. Assn.*, 1913, lxi, 447.

*Experiment 4.*

4 rabbits weighing from 1,900 to 2,200 gm. were given the sediment from 35 cc. of bouillon cultures. 2 minutes later 0.5 cc. of immune serum were injected into the opposite ear vein. 30 minutes later and every 2d hour thereafter 1 cc. of the serum was given. This was continued up to the 56th hour. The final results are shown in Table I.

TABLE I.

Rabbit 1.		Rabbit 2.		Rabbit 3.		Rabbit 4.	
Time.	Observation.	Time.	Observation.	Time.	Observation.	Time.	Observation.
<i>hrs.</i>		<i>hrs.</i>		<i>wks.</i>		<i>hrs.</i>	
67	Right hind leg paralyzed	6	Diarrhea		In a light stupor for 1st day. No other symptoms	50	Both forelegs paralyzed.
83	Both hind legs completely paralyzed; severe opisthotonos; very restless and excitable	24	"			53	Severe opisthotonos, restless, excitable, breathing labored.
96	Comatose; lying on side; breathing labored	48	Diarrhea improved. Rabbit showed no further symptoms	3	Agglutinating titer of serum, 1:125	60	Comatose; lying on side.
122	Died	3	Serum agglutinates pneumococci in a dilution of 1:150	6	Living and in perfect condition	80	Died.
	<i>Autopsy.</i> —Brain and cord intensely injected. Smears from surface of brain and lateral ventricles showed very many pneumococci. No pleurisy, no pericarditis. Blood, lungs, liver, and spleen free of bacteria	6	Living and in perfect condition				<i>Autopsy.</i> —Same as Rabbit 1.

Experiment 4 shows that it is possible by employing properly graduated doses of an immune serum to cure rabbits of a massive pneumococcus infection. The experiment indicates also that it is only the chance diplococci excluded from the influence of the circulating immune serum which escape destruction and multiply. It is of interest to know that the probability of the pneumococci escaping the destructive action of the serum is reduced by one-half by diminishing the infecting dose to a point which is, however, still massive according to the grade of virulence of the strain of pneumococcus employed.

*Experiment 5. A Comparison of the Three Methods of Treatment Employed.*

2 rabbits were used for each method. The infecting dose was the sediment from 20 cc. of bouillon cultures. 2 rabbits were treated, as in Experiment 4,

with frequent small doses of serum; both recovered. 2 were treated first with a small dose of serum and then with a large dose. They died. 2 were given one large dose of serum immediately after the infection; both died of septicemia.

#### DISCUSSION.

The experimental data presented in the foregoing pages should be considered from several points of view: from their bearing on experimental pneumococcus infection in the rabbit, and from their bearing on the theory of the anti-infectious action of immune serum in general.

The most striking fact regarding the experimental pneumococcus infection is the one that a single large dose of the immune serum given at the beginning of the infection is far less effective in overthrowing the infection than small repeated doses of which the maximal amount may not equal the single large quantity unsuccessfully employed.

The reason for this disparity has also been made clear in large part. The serum does not protect by a process of neutralization of a true toxin as antidiphtheritic serum does; or if any neutralization occurs it is a minor, and not the decisive process. The anti-infectious serum performs two things: it brings about an agglutination of the bacteria, and it prepares them for phagocytosis in the organs. Hence, whatever favors this process will be beneficial, and whatever hinders it will be detrimental. A certain concentration of the serum likewise promotes the assembling of the leucocytes in the organs.<sup>1</sup> On the other hand, higher concentration causes the formation of such large clumps as to escape phagocytosis. The free bacteria then quickly multiply, escape into the blood, and cause fatal infection. An excess of serum acts disadvantageously in another way not yet explained. Even when, through a small dose of serum, the small clumps have been formed in the blood and removed by the organs, a large following dose of the serum brings about a fatal issue. In this instance, the bacteria do not begin to multiply and invade the organs. The blood and the organs may be quite or nearly sterile. Death appears to result from intoxication. But just how the serum acts in producing the intoxication has not been determined.

When the pneumococci have all been destroyed in the organs

<sup>1</sup> Bull, *loc. cit.*



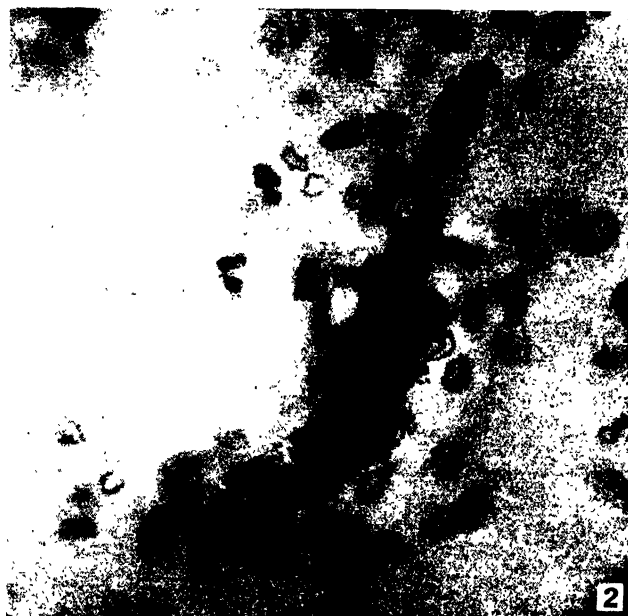
through the operation of the small doses of the serum repeated at intervals, only part of the rabbits are saved. The effect of the serum thus administered is clearly to provoke the destruction of the bacteria under conditions which avoid intoxication. But only those pneumococci are destroyed and rendered harmless that come under the direct influence of the serum which can reach all essential parts of the body except one; namely, the subdural space. When, under any circumstances, the pneumococci reach the subdural space they are not restrained by the treatment but develop rapidly and in great numbers and thus cause a fatal meningitis. The conditions leading to the meningitis are two: (a) large dosage and (b) survival for a sufficiently long period of time. The dose must be larger than the small doses of serum capable of destroying in a certain time period, and the animal must survive even the large doses about 20 hours in order that the meninges may become infected.

But the quantity of virulent pneumococci which rabbits can be made to support under the influence of the method of repeated serum injections is still so very large that the question may be raised whether the limits of activity of this anti-infectious serum have not been greatly underestimated. It remains, of course, to be determined whether still other anti-infectious sera are capable of having their powers enhanced by a similar method of administration. In any case, the subject is one that calls for restudy and perhaps revision.

On the other hand, the experiments affirm nothing as to the efficacy of the method in the serum treatment of lobar pneumonia in man. It may be supposed that so far as the pneumococci in the circulating blood in lobar pneumonia are concerned, small doses of the serum would suffice to bring about their removal. What effect the small doses might have upon the pneumococci in the lungs cannot be predicted. But since the method is one that is readily carried out in man it will doubtless receive attention in due time.

#### SUMMARY.

The treatment of pneumococcic septicemia in the rabbits by large doses of immune serum is detrimental, since the serum causes the



(Bull: Treatment of Pneumococcic Septicemia in Rabbits.)



formation of large clumps of bacteria in the blood which are taken out chiefly by the vessels of the lungs in which they accumulate and impede the circulation.

The large doses of serum are also detrimental when they follow upon small ones through which the small clumps formed are deposited in the spleen, liver, and other organs. In this instance, the large amount of serum leads to the destruction of the pneumococci under conditions which promote an intoxication. The precise mechanism of this action is not known.

The treatment of pneumococcic septicemia in rabbits by small repeated doses of immune serum can be successfully carried out. The number of pneumococci capable of being brought to destruction through phagocytosis in the organs in this way is very great.

Not all the rabbits treated with small repeated doses of the serum survive. Those that succumb do so not to a general infection but to a pneumococcus meningitis. The explanation of this phenomenon is simple. When the number of pneumococci originally inoculated is very great a small number penetrate into the subdural space. Those in this space do not come under the influence of the serum, hence they are not agglutinated and prepared for phagocytosis, whence they multiply and set up a fatal meningitis.

The activity of the immune serum administered in this way against virulent pneumococci is so great that a revision of our notions in the limit of powers of the anti-infectious sera seems necessary. It is patent that the problem is not simply a relation between quantity of immune bodies and number of bacteria. It is more complex than that conception indicates. The factor of the leucocytes and the degree of their possible activities under the conditions of the experiment come into play. Hereafter, in defining the mode and power of action of anti-infectious sera the condition of cooperation of the body-forces will have to be more strictly considered.

#### EXPLANATION OF PLATE 53.

FIG. 1. A large mass of pneumococci in a blood vessel. Tissue was taken from the region of the choroid plexus of a rabbit dying 1 minute after receiving 3 cc. of immune serum. The rabbit had a severe pneumococcus septicemia when the serum was given.

FIG. 2. A large clump of pneumococci. Preparation from the lung of the rabbit described in Fig. 1.

## THE FATE OF TYPHOID BACILLI WHEN INJECTED INTRAVENOUSLY INTO NORMAL RABBITS.

By CARROLL G. BULL, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

### PLATE 54.

(Received for publication, June 1, 1915.)

The course of typhoid bacillus infection in the rabbit has been followed by many investigators. Certain facts are established: The typhoid bacilli injected into the blood do not long remain in the circulation; the gall bladder frequently becomes invaded quickly after the injection; and the bacilli survive and multiply there for a long period of time.<sup>1</sup> Ultimately they may disappear from all parts of the body except the gall bladder.

The present study was undertaken to determine more accurately than had hitherto been done the manner in which typhoid bacilli are removed from the blood of normal rabbits. This study was part of a more general study of the mechanism of bacterial immunity in the rabbit,—whether native or acquired. The rabbit may be regarded as possessing a high degree of natural immunity for the typhoid bacillus, and the fate of the bacilli injected was traced from the blood through the various organs in arriving at an explanation of their disappearance.

### *Source of the Cultures.*

The strain of typhoid bacilli used in the mass of the work was obtained from a capsule of Besredka's sensitized vaccine kindly supplied by Captain H. J. Nichols of the Medical Corps, U. S. Army. Three other typical strains from widely different sources were used to check the results obtained with the Nichols strain. All four strains had been under artificial cultivation for some time and should

<sup>1</sup> Nichols, H. J., *Jour. Exper. Med.*, 1914, xx, 573.

be considered as essentially non-virulent, although death of the animal due to intoxication sometimes ensued. The bacilli used for inoculation were cultivated on plain agar and used when twenty-four hours old. For injections they were washed from the medium with 0.85 per cent sodium chloride solution.

*Disappearance of Bacilli from the Blood.*

From one-thirtieth to one-fiftieth of the bacilli from an agar slant were injected into the ear vein and Petri plates were made from the heart's blood at various intervals, beginning as early as thirty seconds after the injections. It was found that the first specimen taken always contained the largest number of bacilli and that they left the blood with remarkable rapidity. Cultures made ten minutes after the injections contained only a few colonies. Specimens taken fifteen to twenty minutes after injecting the bacteria were often sterile. In some cases a few colonies developed in cultures made several hours after the inoculations or at the time of the death of the animals. Even when the bacilli from an entire agar slant were given and death resulted in two hours the blood was frequently sterile. The following instance will serve as a typical experiment.

*Experiment 1.*—A rabbit weighing 2,000 gm. was given  $\frac{1}{40}$  of a 24 hour agar slant of typhoid bacilli in the ear vein. Blood cultures made from the heart at stated intervals gave the results indicated in Table I.

TABLE I.

Time after Injection, min.	No. of colonies per cc.
1	10,000,000
2	2,500,000
5	100,000
15	40
20	1

In several experiments of this kind the same general results were obtained. In some instances the bacilli left the blood somewhat more slowly than in others, but the variation was a matter of a few minutes only.

The abrupt disappearance of typhoid bacilli from the blood stream was investigated. Why should the bacilli leave the blood

so much more rapidly than other bacteria, namely, streptococci, pneumococci, or dysentery bacilli of the Shiga type, which were studied? If the process was merely a matter of filtration by the capillary systems of the various organs as Wyssokowitsch<sup>2</sup> concluded, this difference should not exist. The notion prevailing has been that the typhoid bacilli are destroyed (dissolved) by the rabbit's blood within a short time. Indeed, it is known that normal rabbit's whole blood or serum *in vitro* kill the bacilli in relatively brief periods of time. Our next inquiry was directed to the organs to determine whether the bacilli taken out of the blood accumulated in them.

#### *Relation of Bacilli to the Tissues.*

From one-thirty-fifth to one-fiftieth of the growth from an agar slant was injected into the ear vein of normal rabbits and specimens of blood were taken for culture from the heart one minute after the inoculation to determine the number of bacilli injected, and again at ten minutes to make sure that the bacilli had left the circulating blood. The rabbits were then killed by a stroke on the neck at ten, twelve, or twenty minutes, and the various organs removed and crushed finely in tissue crushers, after which definite quantities of the pulps were thoroughly shaken in sterile salt solution, and plated. The results of this test are given in illustrative Experiment 2. It may be stated here, however, that in no instance were as many bacilli found in any of the organs per unit of measure as had been present in the blood, and moreover that most of the tissues contained very few bacilli at all.

*Experiment 2.*—A rabbit was given 200,000,000 typhoid bacilli, as determined by test plating, into the ear vein, and the following data were determined: blood from the heart 1 minute after the injection gave 3,000,000 colonies per cc.; 6 minutes after, 80; and 16 and 20 minutes after, no colonies per cc. The tissues removed at 21 minutes gave colonies per cc. of crushed tissue pulp as follows: spleen, 2,000,000; liver, 1,600,000; lung, 100,000; mesenteric lymph node, 1,000; skeletal muscle, brain, and other tissues, comparatively very few. The weights of the organs were determined and the total number of bacteria recovered, with the finding that it was far below the actual number injected. In none of the organs was the number of colonies per unit of measure as great as the number originally found in the blood.

<sup>2</sup> Wyssokowitsch, W., *Ztschr. f. Hyg. u. Infektionskrankh.*, 1886, i, 3.

From this and similarly conducted experiments it was concluded that the bacilli did not accumulate in the tissues, and it was therefore considered desirable to follow the course of events in an organ for several hours after the bacilli had been injected into the blood. The liver was selected for this study since it is possible to remove portions of the organ at various intervals over a period of time. A typical result is given in Experiment 3.

*Experiment 3.*—A rabbit was given  $\frac{1}{8}$  of an agar slant of a culture of typhoid bacilli into the ear vein and estimates were made as follows: Blood 1 minute after the injection gave 15,000,000, and 10 minutes after, 10 colonies per cc.; liver 3 minutes after the injection gave 12,000,000, 14 minutes 6,000,000, 1 hour 700,000, 2 hours 80,000, and 3 hours 1,000 colonies per cc. of crushed pulp.

The data supplied by Experiment 3 support, on first examination, the prevailing notion that certain bacteria and, in this instance, typhoid bacilli fall a ready victim to the destroying and presumably the dissolving effect of the blood, whether in the general circulation or in the capillaries of the organs. This view is based in large part on the classical studies of Wyssokowitsch, and is upheld by the well known fact that fresh serum and the whole blood of rabbits are highly destructive *in vitro* to the typhoid bacilli. Without, however, adopting the usual explanation of the disappearance of the typhoid bacilli from the blood and organs, as illustrated by the experiments on the liver, it was deemed desirable to repeat certain of the experiments on the bactericidal action of normal rabbits' blood.

#### *Action of the Serum and Whole Blood of the Rabbit in Vitro on Typhoid Bacilli.*

*Agglutinins.*—The serum of normal rabbits was tested for agglutinating value. The bacilli were grown upon agar slants and washed off with 5 cc. of normal saline solution. One drop of the suspension of bacilli was added to one cc. of the serum dilution. The agglutinating value of normal serum varied from zero in full serum to positive in 1 to 100 dilution. About one-fifth of the sera examined were devoid of demonstrable agglutinins. The agglutinins are thermolabile and are destroyed at 56° C.

*Bactericidal Power.*—The fresh serum obtained from the heart's



blood after coagulation was diluted with saline solution and from 2,000 to 4,000 typhoid bacilli (estimated by test plating) were added to one cc. of the dilution. The mixtures in test-tubes were incubated at 37° C. for two hours and the entire contents of the tubes were plated. In order to eliminate the clumping of the bacilli and adhesion to the sides of the tubes, melted agar was poured into them after the plating and they were incubated and examined.

The results can be stated as follows: The sera of different rabbits vary considerably in destructive effect on typhoid bacilli. Full serum and serum diluted 1 to 10 usually destroyed all the bacilli. Serum diluted 1 to 50 usually caused diminution but never complete destruction of the bacilli. The defibrinated whole blood and hirudinized blood act in a manner similar to the serum.

There can, therefore, be no doubt that fresh rabbit serum and the whole rabbit blood destroy considerable numbers of typhoid bacilli *in vitro*. Whether the same means operate in the destruction which takes place *in vivo* remains to be determined.

### *Fate of Typhoid Bacilli in the Blood and Organs of Inoculated Rabbits.*

The experiments performed with the pneumococcus<sup>8</sup> suggested that a similar study be made of typhoid bacilli injected into the circulation of rabbits, especially in view of the fact that while rabbits readily succumb to typhoid intoxication they are highly resistant to typhoid infection. Experiment 4 will serve as an illustration of this class of tests.

*Experiment 4.*—An agar slant of typhoid bacilli was suspended in about 5 cc. of salt solution and injected into the ear vein of a normal rabbit. Specimens of the heart's blood were taken and films prepared at 30 seconds, 1, 2, 3, 5, and 7 minutes. The films were stained by Manson's method. Clumps of bacilli were found even in the first film (Fig. 1). The second film showed a larger number of clumps, while the number diminished in the next specimen, and none were found in the last, or 7 minute, specimen. At the end of 7 minutes the rabbit was killed by a stroke on the neck and the organs were immediately removed and finely crushed in tissue crushers. Films were prepared from the pulp and stained by Manson's method. Microscopical examination of the slides showed that clumps of bacilli had accumulated in the capillaries, sinusoids, and blood spaces

<sup>8</sup> Bull, C. G., *Jour. Exper. Med.*, 1915, xxii, 457.

of the various organs, especially the liver, lungs, and spleen, and a large proportion of the clumps had already been phagocyted by the polymorphonuclear leucocytes (Figs. 2, 3, and 4) which had accumulated in the organs following the injection of the bacteria. Free and unclumped bacilli were also found.

This experiment was repeated in its essential aspects several times with wholly concordant results. The experiment was varied in such a manner as to permit the inoculated rabbits to survive for different periods of time, after which the blood and organs were examined. It was found that the largest number of leucocytes enclosing clumps of bacilli were present in the organs of animals killed from thirty to ninety minutes after inoculation. The organs of animals permitted to survive three or four hours still contain many leucocytes, but very few leucocytes containing distinct bacteria are met with. Leucocytes containing granules of disintegrated bacilli or bacilli which have lost power of staining may be found in small number. On the other hand, the three hour specimens still show a certain number of unagglutinated bacilli, outside cells with staining properties unimpaired (Fig. 5).

Still another variation of the experiment was to remove portions of the liver from ten minutes to two hours after injecting the bacilli. The findings just described were corroborated by this procedure. Specimens taken from ten to ninety minutes contained many phagocytizing cells; while later specimens showed fewer phagocytizing cells and finally cells which enclosed only disintegrating bacteria.

#### DISCUSSION.

If we review the findings described in this paper we shall arrive at somewhat conflicting results as to the manner in which typhoid bacilli are disposed of, respectively, by the body and the blood of the rabbit.

Directing attention first to the phenomena observed outside the body it may be affirmed, in keeping with usual knowledge, that the fresh blood serum as well as the fresh whole blood of the rabbit is capable of destroying, apparently by a process of solution, considerable numbers of typhoid bacilli. There is no reason, moreover, to doubt that the process of destruction in this instance is the

common one of bacteriolysis in which amboceptor and complement play the decisive part.

But the essential question at issue is not the extracorporeal but the intracorporeal method of destruction of typhoid bacilli. It is upon that point that light is especially needed. To apply directly the results of test-tube experiments to the explanation of what takes place in the body itself has not proven wholly illuminating. We already know that typhoid bacilli may appear and survive in the blood of human typhoid fever patients at a time when the shed blood is highly bacteriolytic for the bacilli.

The observations which this paper records indicate a wide disparity between the processes involved in the destructions of the bacilli in test-tubes and in the living body. In the latter, the bacilli introduced into the blood are quickly agglutinated, after which they are removed by the organs. In the interstices of the organs they come into close relation with polymorphonuclear leucocytes (themselves assembled in the organs as result of the bacterial injection) which englobe and destroy them. There is no evidence at hand which connects ordinary bacteriolysis with *intra vitam* destruction of typhoid bacilli. The unagglutinated and unphagocyted bacilli in the organs resist longest and stain best; and complement has yet to be detected in the circulating plasma.

This view of the process of *intra vitam* destruction of typhoid bacilli may serve to explain the fact that typhoid bacilli sometimes circulate in the blood of typhoid patients. It is known that bacilli cultivated from the circulating blood are often inagglutinable. We have found no indications that phagocytosis of the bacteria studied by us takes place in the blood or on a grand scale in the unagglutinated state. Hence, as the bacilli cannot be agglutinated and removed by the organs and also cannot be phagocyted in the blood stream, they continue to circulate, under some conditions, until they are removed and destroyed by the phagocytes.

The indications therefore are that in the body the destruction of typhoid bacilli by means of bacteriolysis does not take place. The question arises, however, whether in the tests of the survival of the bacilli in the pulp the conditions produced do not render the operation

of the bacteriolytic processes impossible. To test this point, crushed liver, spleen, and kidney were added to fresh normal rabbit sera just previous to introducing the typhoid bacilli. The test-tubes were incubated for two hours, after which plates were made. It developed that the action of the spleen and kidney pulps was negligible, while the liver pulp caused complete inhibition of bactericidal effect. Further tests indicated that it is the biliary constituent of the liver that is responsible for this action, since bile in quantities themselves non-hemolytic inhibits the activity of a hemolytic system apparently through its anticomplementary effect.

#### SUMMARY.

Typhoid bacilli are agglutinated promptly in the circulating blood of normal rabbits and quickly removed from the blood stream.

The clumped bacilli accumulate in the organs and are taken up by assembled polymorphonuclear leucocytes in the liver, spleen, and possibly other organs.

The phagocytosed clumps of bacilli are digested and destroyed by the phagocytes.

Hence, destruction of typhoid bacilli *intra vitam* is brought about by an entirely different process than is the destruction by serum and whole blood *in vitro*. While the latter is caused by bacteriolysis, the former results from agglutination and intraphagocytic digestion.

Lysis by fresh blood serum is not appreciably affected by spleen or kidney pulp, but it is inhibited by liver pulp. The action of the liver is referable to its biliary constituents, which exert anticomplementary action.

Probably in certain examples of typhoid fever in man the typhoid bacilli in the circulating blood being inagglutinable cannot be removed by the organs and hence are not phagocytosed and destroyed.

The observed disparity between the ready destruction of typhoid bacilli by serum and shed blood and the resistance sometimes offered by the bacilli in the infected body is explained by the essential differences in the destructive processes in operation within and without the body.

EXPLANATION OF PLATE 54.

FIG. 1. Heart's blood from a rabbit containing 2 clumps of typhoid bacilli. The specimen was taken 30 seconds after the bacteria were injected into the ear vein. Stained by Manson's method.

FIGS. 2, 3, and 4. Polymorphonuclear leucocytes from lung, liver, and spleen, respectively, containing clumps of typhoid bacilli. The tissues were removed from the animal 30 minutes after the bacteria were injected. Manson's stain.

FIG. 5. A smear of crushed liver tissue. The specimen was removed from a rabbit 2½ hours after an intravenous injection of typhoid bacilli. (a), leucocyte containing disintegrated bacilli; (b) and (c), free bacilli staining deeply. Manson's stain.



(Bull: Fate of Typhoid Bacilli Injected into Normal Rabbits.)



## THE AGGLUTINATION OF BACTERIA IN VIVO.

By CARROLL G. BULL, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, June 1, 1915.)

Among the factors to which bacterial immunity is ascribed the phenomenon of agglutination occupies the most subordinate place. Indeed, the general view held by immunologists is to the effect that however valuable agglutination may be *in vitro* in identifying bacteria or discovering specific forms of infection, yet the process plays no essential part in the protection of animals as such. The appearance of agglutinins in the sera or body fluids of infected individuals or inoculated animals is considered a sign of the existence of a greater or less degree of immunity; the agglutinins themselves being merely incidental accompaniments of the true immunity factors,—lysins, bacteriotropins, and antitoxins. We have, however, made certain observations within the last few months which indicate that agglutinins play quite an important part in at least certain instances of active and passive immunity.

In reporting his classical work on the specificity and practical application of the phenomenon of agglutination, Gruber<sup>1</sup> expressed the opinion that agglutinins were quite essential properties of an immune serum. He believed that the phenomenon of agglutination was due to the fact that the agglutinins (Glabrificine) increased the viscosity of the bacterial bodies and caused them to adhere to one another. Gruber advanced the idea also that this increased viscosity aided in the englobement of the bacteria by the phagocytes, and would probably explain the accumulation of bacteria in the organs when they were injected into the circulation. Gruber's hypothesis has never been further developed or proved by himself or others.

Metchnikoff<sup>2</sup> contends that agglutinins play no rôle, however small, in active or passive immunity processes. "The phenomenon of agglutination is of no great importance from the point of view of natural immunity."<sup>3</sup> "We have already given the arguments which render it impossible for us to attribute to the agglutinative property of the fluids of the body any rôle, however unimportant,

<sup>1</sup> Gruber, M., *Wien. klin. Wchnschr.*, 1896, ix, 183, 204.

<sup>2</sup> Metchnikoff, E., *Immunity in Infective Diseases*, Cambridge, 1905.

<sup>3</sup> Metchnikoff, *loc. cit.*



in natural immunity against micro-organisms."<sup>4</sup> "The part played by agglutination in this immunity (acquired) is merely accidental and subordinate."<sup>5</sup>

After extensive investigation, Salimbeni<sup>6</sup> concluded that bacterial agglutination never takes place within the animal organism. This author worked with animals immunized to the cholera vibrio. The sera of the animals were strongly agglutinative *in vitro*, but he was unable to find any evidence indicative of agglutination *in vivo*.

Sawtschenko and Melkich<sup>7</sup> believed that agglutinins were present and active in the plasma of cases of recurrent fever. They found small clumps of spirochætes in blood immediately after its removal from the patients. It was suggested by them that the agglutination in the plasma was incomplete because of the rapid movement of the blood.

A glance at the literature given above readily convinces one of the slight consideration agglutinins as protective agents have received. Gruber's suggestions seem to have gone by as such and Sawtschenko's observations have not been considered sufficient proof of the functioning of agglutinins *in vivo*. Salimbeni has done the most direct and extensive work on this subject and his results led him to conclude that agglutination is strictly a phenomenon of the test-tube. And Metchnikoff is quite firmly convinced of the unimportance of agglutinins in both natural and acquired immunity. Our observations made in a more or less incidental manner seem to justify a different conception of agglutination as an immunity factor, for which reason they have been put together in this paper.

### *Pneumococci and Agglutination in Vivo.*

In the course of the study of the manner of the rapid disappearance of pneumococci from the blood stream of rabbits following the injection of antipneumococcus serum the observation was made that an almost instantaneous agglutination of the pneumococci was produced in the blood by the serum introduced and, furthermore, that the clumped or massed cocci quickly accumulated in the internal organs.<sup>8</sup>

These facts in themselves are not only new, as is evident from the literature previously quoted, but they are important as bearing on our conception of the biological processes which come into play in the course of bacterial immunity. Aside from this consideration, however, the facts are of interest in connection with the following points: (1) the time required for the reaction to take place; (2) the

<sup>4</sup> Metchnikoff, *loc. cit.*, p. 258.

<sup>5</sup> Metchnikoff, *loc. cit.*, p. 263.

<sup>6</sup> Salimbeni, A. T., *Ann. de l'Inst. Pasteur*, 1897, xi, 277.

<sup>7</sup> Sawtschenko and Melkich, *Ann. de l'Inst. Pasteur*, 1901, xv, 497.

<sup>8</sup> Bull, C. G., *Jour. Exper. Med.*, 1915, xxii, 457.

degree of its specificity; (3) the concentration of serum necessary to effect agglutination; and (4) the relation of the quantity of serum employed to the course of a bacterial septicemia.

#### EXPERIMENTAL.

When the agglutination tests are made in test-tubes it is customary to incubate the tubes for two hours at 37° C. and then to set them aside at a low temperature for another period of several hours before the results are read.

In contradistinction to the slowness with which agglutination occurs and becomes evident *in vitro* is its instantaneous occurrence *in vivo*. To demonstrate this point we have proceeded as follows: The bacteria (pneumococci) from 50 cc. of bouillon are injected into the ear vein of a rabbit, after which a specimen of blood is taken from the heart to ascertain if sufficient bacteria are present to be easily found with the microscope and to determine the absence of clumps. The antiserum is then injected and a specimen of blood taken from the heart not later than thirty seconds afterwards. Other specimens are taken at one, two, three, five, and ten minute intervals. The sample of blood removed at twenty seconds usually contains the largest and greatest number of clumps. The second sample may also show many clumps of fair size, but the later samples show only a few small clumps. As a rule, no clumps or free bacteria whatever can be found at the expiration of five or ten minutes, particularly if from 1 to 2 cc. of serum have been injected.

Why bacteria agglutinate so much more rapidly *in vivo* than *in vitro* is not evident. The temperature conditions do not offer a satisfactory explanation, because the reaction does not occur in the test-tube immediately after body temperature is reached. The effect of the agitation caused by the circulation of the blood is not known, but would not seem to suffice to produce the great differences noted. It is quite possible that some constituent of the blood of the host aids the specific serum in producing those changes in the bacterial bodies which precede agglutination, but of this we are wholly ignorant.

The specificity of antipneumococcic agglutinins is as strict *in vivo* as *in vitro*. A heterologous serum causes no agglutination in a

concentration fifty times as great as that in which a homologous serum gives a positive reaction.

In following the reaction quantitatively *in vivo* we found that quantities of serum as small as 0.05 cc. per kilo of body weight of the rabbit caused the formation of small clumps, and that that quantity was the least amount that exercised any appreciable influence on the course of the septicemia or sufficed to prolong the life of the inoculated animals. Since the agglutinating titer of the anti-serum employed was 1 to 50 by the macroscopic method, this result was wholly unexpected. But the result was rendered more comprehensive when it was ascertained that by merely modifying the method somewhat the titer could be increased to 1 to 500. Thus, when from 0.08 to 0.1 cc. of a bouillon culture of the pneumococci is added to 1 cc. of the diluted serum, clumps are formed which may be large enough to be seen by the naked eye, provided the fluids are clear. Stained preparations viewed with the microscope also reveal the clumps. But even so, agglutination within the body seems to take place with dilutions of the serum which are wholly ineffective in the test-tube.

#### *Typhoid Bacilli and Agglutination in Vivo.*

When typhoid bacilli are injected into the circulation of normal rabbits, they quickly leave the blood. The precise manner of the disappearance has not been investigated. Because the typhoid bacilli are subject to lysis it has been supposed that they are in fact disintegrated.

When, however, the blood was examined as described in connection with the pneumococci it was learned that the bacilli agglutinate within a few seconds after entering the blood stream. The rapidity with which agglutination takes place is affected by the power of the serum to cause agglutination in the test-tube. With rabbits whose blood does not possess this power a single bleeding could be performed before clumps were formed; in all others, agglutination had taken place within the brief period of thirty seconds.

The heating of typhoid bacilli to 80° C. for half an hour rendered them more readily agglutinable *in vivo* than unheated bacilli. While bacilli heated to from 65° to 80° C. proved inagglutinable in test-

tubes, our experience here confirmed the observation of others.<sup>9</sup> In this respect, therefore, agglutination *in vivo* differs from that *in vitro*.

#### *Dysentery Bacilli and Agglutination in Vivo.*

As a rule, normal rabbit serum does not agglutinate *in vitro* in dilutions of 1 to 10 any of the varieties of dysentery bacilli. The sera of all the rabbits we tested were inactive in this dilution. We employed the following strains: Shiga, Strong, Hiss, and Flexner.

The strains of the Shiga type do not agglutinate and remained in the blood for twenty minutes distributed uniformly; while the other strains undergo immediate agglutination, clumps being found twenty seconds after the bacteria were injected. By means of this reaction it was possible to determine within two minutes whether the culture tested belonged to the Shiga or Flexner groups of dysentery bacilli.

The fate of the dysentery bacilli could also be followed. When the rabbits are killed as early as seven minutes after receiving an inoculation of one of the Flexner group of bacilli, large numbers of leucocytes carrying clumps of one hundred or more bacilli were already present in the lungs, liver, and spleen.

When an immune serum for the Shiga type of bacillus is injected into the circulation the Shiga bacilli, which otherwise do not agglutinate, become immediately clumped. The distinction, therefore, in the behavior of the Shiga and the Flexner groups depends on the presence in the normal rabbits of agglutinins for the one and not for the other group.

#### *Bacillus influenzae and Agglutination in Vivo.*

A non-virulent strain of *Bacillus influenzae* isolated from the respiratory tract agglutinated in the circulation of normal rabbits one minute after injection. A virulent strain isolated from a case of influenzal meningitis was not agglutinated and was still in the circulating blood twenty minutes after injection. The distinction of virulent and non-virulent strains is determined by the fact that the former causes a fatal septicemia in young rabbits, while the latter does not.<sup>10</sup>

<sup>9</sup> Porges, O., *Ztschr. f. exper. Path. u. Therap.*, 1905, i, 621.

<sup>10</sup> Wollstein, M., *Jour. Exper. Med.*, 1915, xxii, 445.

## DISCUSSION.

The results described in the preceding pages emphasize the occurrence as well as the significance of agglutination of bacteria in the blood once they have gained access to the circulation.

In the first place, the power of the blood in normal animals to cause agglutination determines, apparently, in large measure whether the bacteria are to be promptly removed and septicemia avoided or to remain and to bring about without restraint that serious condition. The importance of this power as far as the normal rabbit is concerned is well illustrated by the examples afforded, on the one hand, by pneumococci, dysentery bacilli of the Shiga type, and virulent influenza bacilli, and, on the other hand, by typhoid bacilli, dysentery bacilli of the Flexner group, and non-virulent influenza bacilli.

The normal blood of the rabbit fails to agglutinate the Shiga dysentery bacilli outside (*in vitro*) or even inside the circulation, with the result that they are not converted into clumps in the blood stream and hence are not promptly removed from it, and while the normal blood does not agglutinate the Flexner group of dysentery bacilli in the test-tube in a 1 to 10 dilution even after two hours' incubation at 37° C., it does agglutinate them immediately in the circulation, whence they are quickly removed. Similarly, the circulating blood of the rabbit does not agglutinate pneumococci or the virulent form of influenza bacilli, with the result that they remain and multiply there, leading to a septicemia to which the animal succumbs; while typhoid bacilli and the non-virulent form of influenza bacilli are instantly agglutinated and promptly removed from the circulation.

In view of these facts we can discern in the phenomenon of agglutination in the blood an essential mechanism through which bacteria are so changed that they accumulate in the capillaries and sinuses of the viscera; and perceive in this mechanism the decisive act which determines whether protection is to be afforded or fatal septicemia supervene.

Indeed, the facts at hand illuminate the mechanism of protection from infection even further. It would appear that in the instances given the bacteria neither accumulate in the organs to any degree

when unagglutinated nor are they taken up by the leucocytes within the circulation when they remain there. Moreover, those bacteria which enter the organs before agglutination takes place remain in the single form and escape phagocytosis. When, however, agglutination has taken place the bacteria are quickly removed from the circulation and are englobed by leucocytes in the lungs, liver, spleen, and other organs, and, as may be inferred from the rapidity with which they are disintegrated in the phagocytes as compared with their persistence when free, are quickly destroyed, and possibly finally detoxicated. In other words, in order that the bacteria may be promptly removed from the blood stream it is requisite that they be first agglutinated, which condition is also required in order that they be destroyed *en masse* within the organs, a process achieved, apparently, chiefly through phagocytosis.

This phenomenon of protection in the normal animal is paralleled by what happens when an effective antiserum is employed to prevent or combat a bacterial infection, as is illustrated by the examples given of serum protection in pneumococcus and Shiga bacillus infection in the rabbit. It may be inferred that similar processes occur in other infections and in other animals, including man, but many more particular cases will need to be investigated before general deductions are made.

#### SUMMARY.

1. Small quantities of antiserum bring about instantaneous agglutination of pneumococci in the circulation of the rabbit; the reaction is specific and occurs in every case in which sufficient serum is given to influence the course of the septicemia or to prolong the life of the animal.

2. The agglutinating titer of antipneumococcus serum can be made considerably higher by adding only a small quantity of culture to the tests, thus making the test a finer differential.

3. Typhoid bacilli agglutinate spontaneously in the circulation of the normal rabbit; the reaction is positive *in vivo* even in cases in which undiluted serum gives a negative result *in vitro*; heating the bacilli to 80° C. for thirty minutes renders them more agglutinable *in vivo*.

4. Dysentery bacilli of the Shiga type do not agglutinate in the blood stream of the normal rabbit, but a small quantity of antiserum injected into the circulation causes immediate agglutination; while all strains of the Flexner group undergo spontaneous agglutination.

5. Non-virulent influenza bacilli agglutinate spontaneously in the circulation of the normal rabbit; virulent strains remain in the blood unclumped.

6. In all instances so far investigated of both passive and natural immunity, agglutination of the bacteria within the blood of the infected animal was followed by a rapid removal of the bacteria from the circulation, and by phagocytosis and destruction of the agglutinated bacteria in the capillary systems of the viscera; while those bacteria which are not agglutinated remain in the circulation and produce a progressive septicemia.

7. Hence the agglutinins seem to play the decisive part in at least certain instances of bacterial infections.

## CHEMOPATHOLOGICAL STUDIES WITH COMPOUNDS OF ARSENIC.

### I. TYPES OF THE ARSENIC KIDNEY.

BY LOUISE PEARCE, M.D., AND WADE H. BROWN, M.D.

*(From the Laboratories of The Rockefeller Institute for Medical Research.)*

PLATES 57 TO 60.

(Received for publication, May 15, 1915.)

During the past year, while investigating the pathological action of a large number of organic preparations of arsenic,<sup>1</sup> it was noted that the gross appearance of many of the kidneys resulting from the administration of these compounds did not conform to the type usually described as the arsenic kidney. The wide variations in the type of kidney observed, and at the same time the constancy of the type produced by a given compound in a given animal species, were so marked as to suggest that the classical picture was not a constant one for all compounds of arsenic. To obtain a more comprehensive idea of these variations and some conception of their cause, a series of experiments was carried out with a number of well known arsenicals, and the gross pathological results obtained serve as the basis for this report. The microscopical studies are reported in a separate paper.

#### EXPERIMENTAL.

Dogs were used for these experiments, because the dog kidney is large enough to show the gross pathological picture quite well. This series of experiments was supplemented, however, by similar experiments carried out with guinea pigs, intended principally as a

<sup>1</sup>For several years, The Rockefeller Institute for Medical Research has been interested in the chemotherapy of infections. The work now in progress is being carried out jointly by a chemical division and a biological division. The chemical work is being done by Dr. W. A. Jacobs and Dr. Michael Heidelberger. The series of studies contemplated under the title "Chemopathological Studies with Compounds of Arsenic" are an outgrowth of the work on chemotherapy.



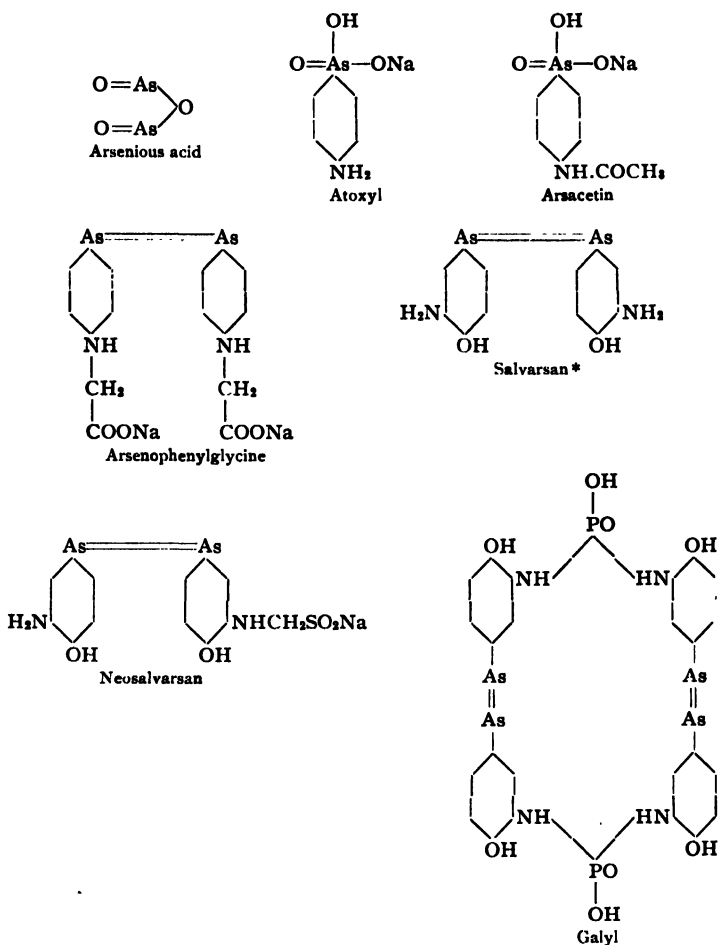
control upon the histological changes produced, since the dogs obtainable for this work so frequently showed spontaneous kidney lesions.<sup>2</sup> It may be stated here that the gross appearances presented by the guinea pig kidneys were essentially the same as those of the dogs.

TABLE I.

Dog.	Drug.	Dose per kilo.	Strength of solution.	Route of administration.	Remarks.					
					1 day.	2 days.	3 days.	4 days.	5 days.	6 days.
A	Arsenious acid	7	1:100	Subcutaneous	Dead					
B	Arsenious acid	5	1:333	Intraperitoneal	"					
C	Atoxyl	40	1:50	Subcutaneous	Slightly ill	Well	Well	Weak	Better	Dead
D	"	20	1:10	Subcutaneous	Well	"	40 mg.	Ill	Killed	
E	"	50	1:50	Intraperitoneal	Ill; killed					
F	"	20	1:75	Intraperitoneal	" "					
G	Arsacetin	300	1:10	Intraperitoneal	Ill	Better	Better	Well 600 mg.	Dead	
H	"	400	1:25	Intravenous	Ill; killed					
I	"	600	1:10	Intravenous	" "					
J	"	400	1:40	Intravenous	Ill	Dead				
K	Arsenophenylglycine	225	1:10	Intraperitoneal	"	Better	3 days. Well 225 mg.	19 days. Well 250 mg.	20 days. Well; killed.	
L	Arsenophenylglycine	500	1:10	Intravenous	Dead					
M	Arsenophenylglycine	300	1:20	Intravenous	"					
N	Arsenophenylglycine	200	1:20	Intravenous	Well; killed					
O	Arsenophenylglycine	500	1:15	Intravenous	Dead					
P	Salvarsan	100	1:400	Intraperitoneal	Ill	Ill; killed				
Q	"	100	1:333	Intravenous	Dead					
R	Neosalvarsan	150	1:30	Intravenous	"					
S	Galyl	100	1:100	Intravenous	"					

<sup>2</sup> Dayton, H., *Jour. Med. Research*, 1914, xxxi, 177.

TABLE II.  
*Structural Formulae of Arsenicals.*



\* Preparation No. 606 in Ehrlich's series designated as salvarsan is the dihydrochloride of this compound. The formula here given, however, is that most frequently encountered in the literature and is the substance derived from the dihydrochloride by the addition of alkali.

Solutions of arsenious acid, atoxyl, arsacetin, arsenophenyglycine, salvarsan, neosalvarsan, and galyl were injected without an anesthetic into young adult dogs. The injections were made intravenously, intraperitoneally, or subcutaneously; no difference was

noted in the results obtained by using these different routes. Doses of various size were used, as shown in Tables I and II, but in the majority of instances amounts approximating the lethal dose were injected, in order to accentuate the lesion produced by the particular compound. In a few instances much smaller amounts were used, in order to determine the point of greatest susceptibility and the characteristics of the essential lesions. The animals were usually killed at the end of twenty-four hours, if they were not already dead, so that we might obtain lesions comparable in time (length of survival) as well as dose. In each instance the dog was lightly anesthetized with chloroform and bled to death from the carotid artery.

#### RESULTS.

##### *Arsenious Acid.*

Injection of arsenious acid into dogs produces a uniformly red kidney (Fig. 1). The organ is enlarged, is purplish red in color, and on section drips blood. The cut surface is reddened from the outermost edge to the pelvis, through the cortex, boundary zone, and medulla. All the zones or territories of the kidney are practically uniformly involved. The kidneys of dogs that survive large doses of arsenious acid for a sufficient length of time show opaque, yellowish white striations in the inner portion of the cortex resulting from tubular necrosis which, however, is not an essential feature of the change. The outspoken picture is that of a diffusely reddened kidney indicative of uniform vascular injury and is one of the two principal types produced by arsenical compounds.

##### *Salvarsan, Neosalvarsan, and Galy.*

The type of kidney produced by injections of these drugs falls into the general class of red kidneys, although certain differences relate them to the other main type, the pale kidney. These differences consist in a relatively greater degree of tubular necrosis and in the character of the vascular injury. This group of kidneys, as shown by the salvarsan and neosalvarsan kidneys (Figs. 3 and 8, respectively), shows a considerable amount of necrosis of the cortex which is most conspicuous in the inner half. On the other hand,

although the kidney is reddened throughout, the congestion and hemorrhage are most prominent in the outer half of the cortex and the inner portion of the boundary zone.

#### *Arsacetin.*

Injection of arsacetin into dogs produces an exceedingly pale kidney (Fig. 2), that differs strikingly from the red type just described. The organ is enlarged, soft, and very friable. It is grayish or yellow in color; in some instances it may be pink, with slight congestion of the surface vessels. It cuts very easily and on section the cut edge is everted. The cortex bulges, is widened, and the striations are yellowish, opaque, and raised as a result of wide-spread tubular necrosis; the glomeruli may or may not be prominent. The boundary zone practically always shows some congestion and the same may be said of the medulla, but to a much less degree. The dominant feature of this type of kidney, therefore, is its paleness due to tubular necrosis, as contrasted with the redness of the kidney of arsenious acid resulting from vascular injury. Kidneys of dogs that survive large doses of arsacetin for a sufficient length of time show a greater degree of congestion or even some hemorrhage in the boundary zone. The hemorrhage may extend somewhat into the medulla and to a lesser degree into the cortex.

#### *Arsenophenylglycine and Atoxyl.*

Both of these drugs produce pale types of kidneys, but the amount and extent of vascular injury are somewhat greater than in the arsacetin kidney. The cortical necrosis of the arsenophenylglycine kidney (Fig. 4) is extreme and is quite comparable to that produced by arsacetin. On the other hand, the congestion and hemorrhage of the medulla are relatively prominent and especially so in the inner portion of the boundary zone. We may, therefore, consider the kidneys produced by these drugs as related to both the pale and the red types of kidneys.

#### DISCUSSION.

In studying the action of this group of arsenicals upon the kidney, the object in view has necessitated the subordination of detail to the

broader conception of types of renal injury. Further, we can not now attempt a complete description and grouping of all the types of arsenic kidneys which we have observed. Our object, at present, is to lay emphasis upon the difference in the character of the renal injury produced by different compounds of arsenic as expressed in the gross appearance of the organ. For this purpose we have chosen two extreme types.

The classical red kidney resulting from injections of arsenious acid into dogs contrasts sharply with the pale kidney produced by arsacetin. The red kidney is one in which uniform vascular injury predominates; all zones of the kidney seem to be affected equally, cortex as well as medulla. The pale kidney, on the other hand, presents striking evidence of the dominance of tubular necrosis, and the reddening of the boundary zone is a relatively inconspicuous feature. With the other arsenicals this difference in the gross appearance of the kidneys is not so marked, and we may perhaps look upon the kidneys produced by salvarsan, neosalvarsan, and galyol, on the one hand, and by atoxyl and arsenophenylglycine on the other, as constituting transitions or subgroups between the two principal types. The salvarsan group of kidneys is preeminently red, but there is also a relatively great degree of tubular necrosis; the arsenophenylglycine group of kidneys is pale, yet it shows relatively marked vascular injury, especially in the boundary zone and contiguous territory.

The type of kidney produced by a given compound can not be regarded as fixed in all its details, but the size of the dose and the rate of action as measured by the length of survival of the animal are factors that exercise a distinct influence upon the final result, as may be illustrated by atoxyl. The lethal effects of atoxyl are developed slowly in the dog and it would probably require many times the minimal lethal dose to kill within twenty-four hours. The tolerable dose of this drug for dogs as given by Mesnil and Brimont<sup>3</sup> is 10 mg. per kilo of body weight. Dog F, given double this dose (20 mg. per kilo), showed slight symptoms of intoxication at the end of twenty-four hours and when killed the kidneys were quite pale and slightly enlarged. The cortex showed opaque striations,

<sup>3</sup> Mesnil, F., and Brimont, E., *Ann. de l'Inst. Pasteur*, 1908, xxii, 856.

but the striking feature was a pencil line of hemorrhage in the inner boundary zone (Fig. 6). This animal illustrates well the character and location of the essential changes produced by atoxyl. Dog E, given 50 mg. of atoxyl per kilo, was more toxic at the end of twenty-four hours and was killed. The kidneys (Fig. 7) were markedly enlarged and the opaque yellow gray surface was thickly stippled with red. On section the cortex showed alternate striations of red and opaque yellowish gray; the glomeruli were prominent. The line of red in the medulla of Dog F had given place to an intense reddening of the entire medulla, which was practically a mass of clotted blood. The massive dose of atoxyl used in this dog exaggerates the effects produced in Dog F and at the same time serves to indicate the character of changes referable to dosage. Dog C received 40 mg. of atoxyl per kilo and although this animal passed large amounts of blood in the urine, other evidence of intoxication was relatively slight. It finally died on the sixth day. The kidneys of this dog were practically identical with those of Dog D (Fig. 5) which received a dose of 20 mg. per kilo, three days later a second dose of 40 mg. per kilo, and was then killed after two days. With these two dogs the influence of time as well as dose is introduced. This group of atoxyl kidneys illustrates well the elasticity of a type but at the same time emphasizes the sharp differentiation of even a transitional group from the classical arsenious acid kidney.

As regards the factors that endow compounds of arsenic with the power of producing constant and characteristic types of renal injury, one can hardly escape the conclusion that such activities are intimately related to the chemical constitution of the compounds. However, both organic and inorganic compounds of arsenic in which the arsenic is trivalent or pentavalent are known to produce red kidneys of the arsenious acid type. On the other hand, while both trivalent and pentavalent compounds of arsenic produce pale kidneys, we know of no inorganic arsenical that acts in this way. Further, substances as closely related as atoxyl and arsacetin, or as salvarsan and arsenophenyglycine, may act quite differently. Again, compounds as different as arsenious acid and salvarsan, or as arsacetin and arsenophenyglycine, may simulate each other quite closely in their effects.

For the present, therefore, we can go no further than the conclusion that the character of the renal injury produced by compounds of arsenic is determined either directly or indirectly by the chemical constitution of the compound.

#### CONCLUSIONS.

1. All arsenical compounds do not produce the same type of renal injury.

2. In general, there are two broad groups of kidneys produced, the red and the pale, with a variety of subdivisions of each group depending upon modifications in the chemical constitution of the compound, dosage, and length of survival of the animal.

#### EXPLANATION OF PLATES.

##### PLATE 57.

FIG. 1. Kidney of arsenious acid (natural size). Dog A. Red type of kidney. Uniform congestion and hemorrhage throughout cortex and medulla. Tubular necrosis in inner half of cortex.

FIG. 2. Kidney of arsacetin (natural size). Dog I. Pale type of kidney. Necrosis of cortex. Congestion of boundary zone.

##### PLATE 58.

FIG. 3. Kidney of salvarsan (natural size). Dog Q. Reddening of kidney throughout, especially of outer cortex and inner portion of boundary zone. Tubular necrosis of inner half of cortex.

FIG. 4. Kidney of arsenophenylglycine (natural size). Dog L. Tubular necrosis of entire cortex. Diffuse congestion of medulla with a band of hemorrhage.

##### PLATE 59.

FIG. 5. Kidney of atoxyl (natural size). Dog D. Necrosis of cortex. Diffuse congestion of medulla with hemorrhage of boundary zone and inner half of cortex.

##### PLATE 60.

FIG. 6. Kidney of atoxyl (reduced). Dog F. Pencil line of congestion and hemorrhage along inner boundary zone.

FIG. 7. Kidney of atoxyl (reduced). Dog E. Necrosis of cortex. Hemorrhage of medulla.

FIG. 8. Kidney of neosalvarsan (reduced). Dog R. Necrosis of cortex. Congestion and hemorrhage of boundary zone.

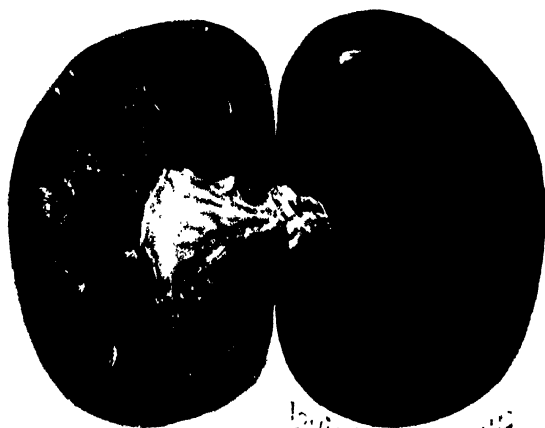


FIG. 1.

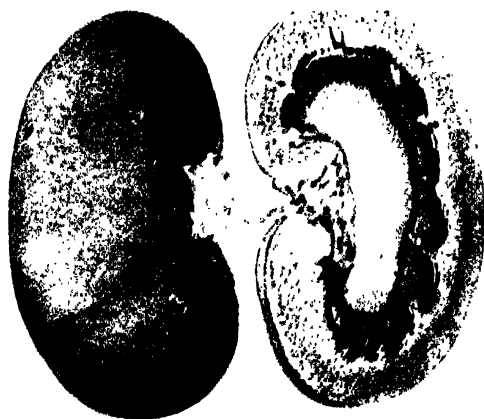


FIG. 2.

(Pearce and Brown: Types of Arsenic Kidney.)





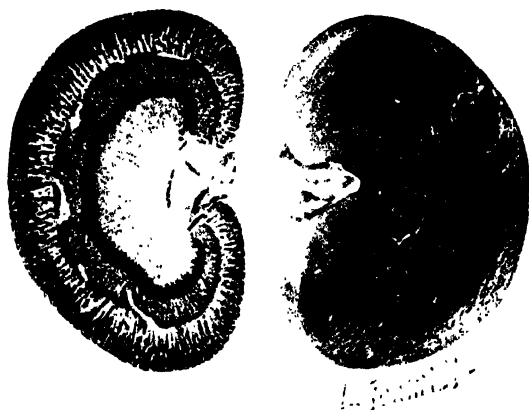


FIG. 3.

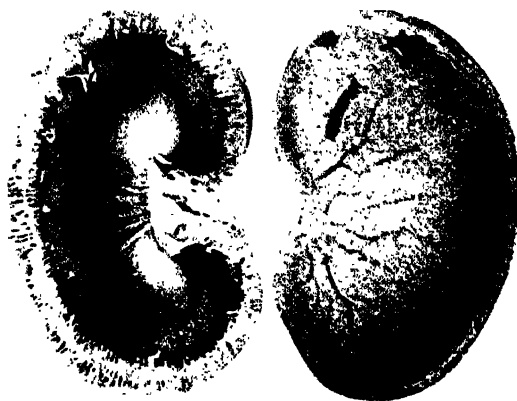


FIG. 4.

(Pearce and Brown: Types of Arsenic Kidney.)





FIG. 5.

(Pearce and Brown: Types of Arsenic Kidney.)





FIG. 7.



FIG. 8.

(Pearce and Brown: Types of Arsenic Kidney.)



## CHEMOPATHOLOGICAL STUDIES WITH COMPOUNDS OF ARSENIC.

### II. HISTOLOGICAL CHANGES IN ARSENIC KIDNEYS.

BY LOUISE PEARCE, M.D., AND WADE H. BROWN, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 61 TO 67.

(Received for publication, June 24, 1915.)

The idea that arsenicals as a class produce a vascular type of injury in the kidney has gained general acceptance, and workers in experimental nephritis have substantiated this idea for such compounds as have been employed to produce so called arsenical nephritis. However, as we have shown in the preceding paper,<sup>1</sup> all compounds of arsenic do not produce the same type of injury, and from the gross appearance of the kidneys of dogs poisoned with various well known arsenicals, we are able to recognize two widely separated types of kidney, the red and the pale, with many transitional varieties or subgroups, each being more or less characteristic for a given compound. In like manner, we have found that the histological changes in these given types are equally different and characteristic.

#### EXPERIMENTAL.

Tissues from the kidneys described and illustrated in the preceding paper<sup>2</sup> were fixed in Zenker's fluid, sectioned in paraffin, and stained with hematoxylin and eosin. The histological changes, therefore, may be related directly to the descriptions and illustrations in this paper.

#### *Red Kidneys.*

*Arsenious Acid.*—The most characteristic acute lesions produced by arsenious acid consist in a uniform dilatation and congestion of

<sup>1</sup> Pearce, L., and Brown, W. H., *Jour. Exper. Med.*, 1915, xxii, 517.

<sup>2</sup> Pearce and Brown, *loc. cit.*



the blood vessels of the kidney with an escape of blood into the interstitial tissues throughout the cortex and medulla (Fig. 1). The glomeruli are swollen, and the tuft completely fills the capsular space. Occasionally there is desquamation of swollen, disintegrating cells of the capsular epithelium and a slight albuminous precipitate in the capsular space. The tuft vessels are widely dilated and filled with blood; some contain hyaline plugs. The changes in the tubular epithelium are less marked, the cells are greatly swollen and vacuolated, often occluding the lumen of the tubule and are frequently stripped up from the basement membrane. Parenchymatous and fatty degeneration are marked, but necrosis of tubular epithelium is relatively slight. Some of the tubules contain an albuminous precipitate; others, especially in the boundary zone, contain blood.

*Salvarsan.*—While vascular injuries dominate the histological changes produced by poisoning with salvarsan, the lesions differ in essential respects from those produced by arsenious acid. Throughout the cortex and medulla the vessels are dilated and congested, but patches of hemorrhage are more numerous in the outer cortex and are especially prominent in the boundary zone. The glomeruli are all large, with the tuft practically filling the capsular space; the tuft capillaries are widely dilated and filled with blood; and there is a slight accumulation of polymorphonuclear leucocytes in the capillaries. The cells of the outer portion of many of the tufts are markedly swollen, granular, and pale-staining, and many of these swollen cells appear to be desquamating into the capsular space (Fig. 4). Hyaline thrombi are numerous (Fig. 3). Occasionally there is a slight amount of albuminous precipitate in the capsular space. The changes in the tubules are relatively more pronounced than with arsenious acid. The epithelium of the tubules shows an extreme degree of parenchymatous and fatty degeneration with definite necrosis. The cells are very granular and ragged with frayed edges, and many are actually disintegrating (Figs. 3 and 4). In many areas there is a protrusion of swollen tubular epithelium into the glomerular space. Practically all the tubules contain albuminous precipitate and some of the cortical and many of the medullary tubules contain blood (Fig. 3). Of especial interest is a fairly extensive and regular edema of the labyrinth, particularly of the

lower two-thirds of the cortex extending but little into the medulla (Fig. 4).

*Neosalvarsan*.—The histological changes produced in the kidney by neosalvarsan are still further removed from those produced by arsenious acid, both in type and degree, although they conform in general to the changes observed in other red kidneys. Here again vascular dilatation and congestion are present, and hemorrhage, as with salvarsan, is most marked in the outer cortex and boundary zone.

The glomeruli are large, but the tuft itself is small and compressed, occupying approximately only one-third to one-half of the capsular space. The remaining space is filled with an extensive albuminous precipitate (Fig. 5). The tuft vessels are irregular, many being enormously dilated and filled with blood and numerous hyaline plugs; in consequence, other vessels are completely collapsed. There is an increase in the number of polymorphonuclear leucocytes in the capillaries of the tuft. The injury to the tubular epithelium is distinctly more prominent than with either arsenious acid or salvarsan. The epithelium of the convoluted tubules, particularly of the outer half of the cortex, is almost completely necrotic, the epithelium of many of the tubules being converted into homogeneous, pink-staining masses (Fig. 5). There is less tubular necrosis in the inner cortex, but there is marked parenchymatous and fatty degeneration with an albuminous precipitate in the lumen of the tubules. A considerable number of the ascending limbs of the loops of Henle shows an extreme degree of degeneration occasionally going on to necrosis. Many of the tubules in the boundary zone and medulla contain blood. In the lower portion of the cortex and in the boundary zone there are a few irregular patches of interstitial edema.

*Galyol*.—In general, sections from kidneys after injection of galyol resemble those of salvarsan and neosalvarsan, but there are certain relative differences. The tuft capillaries are uniformly dilated and contain blood and irregularly distributed hyaline thrombi. There is a considerable amount of swollen cells desquamated from the capsule into the capsular space (Fig. 2). The epithelium of the convoluted tubules, especially in the outer half of the cortex, shows disintegration, desquamation, and necrosis, but there is very little necrosis *en masse*. The epithelium of the loops of Henle shows de-

generation, although comparatively little necrosis. The cortical tubules contain much albuminous precipitate, and the majority of the tubules of the medulla contain blood.

### *Pale Kidneys.*

*Arsacetin.*—In the group of pale kidneys, of which the arsacetin kidney is taken as the type, the relative degree of vascular and tubular injury is the reverse of that observed in the red kidney. Following the injection of arsacetin, the vessels and capillaries of the kidney are not usually dilated or congested except to a slight degree in the boundary zone, and there is no hemorrhage. The glomeruli are practically normal in appearance; the tuft fills most of the capsular space but is not swollen; the capillaries of the tuft are moderately dilated and contain some blood. There is only a slight amount of albuminous exudate. On the other hand, practically all the epithelium of the convoluted tubules is necrotic, the tubules appearing as large, swollen, granular, pink-staining masses (Fig. 6). The cells which are not actually necrotic are extremely degenerated with pyknotic and fragmenting nuclei. The tubules are choked with masses of disintegrating cells or contain an albuminous precipitate. The epithelium of the loops of Henle is markedly degenerated, the cells are swollen, ragged, and vacuolated. In some instances, when a very large dose of arsacetin is given (Dog I),<sup>8</sup> the wide-spread necrosis of tubular epithelium includes that of the ascending limb of the loop of Henle. The collecting tubules show a moderate amount of parenchymatous and fatty degeneration.

*Arsenophenylglycine.*—The injury produced in the kidney by arsenophenylglycine combines both vascular and tubular changes. The vessels and capillaries throughout the kidney are moderately dilated and congested. There is slight escape of blood, if any, from the vessels in the cortex, but in the boundary zone there is a considerable amount of hemorrhage which extends in streaks into the medulla. The glomeruli are uniformly large, the tuft filling approximately one-third to one-half the capsular space. The capillaries of the tuft are irregular in appearance; some are moderately

<sup>8</sup> The designations of the dogs correspond to those given in the preceding paper (Pearce and Brown, *loc. cit.*).

dilated and filled with blood, but many are completely collapsed. The epithelium of the capsule and of the adjoining portion of the connecting tubule is enormously swollen, homogeneous, and pale pink. Many cells of the capsule and connecting tubule have desquamated into the capsular space (Fig. 7), and in certain instances the mass of degenerated and disintegrating cells in the space apparently comes almost entirely from the tubule. Practically all the convoluted tubules are necrotic and appear as solid, homogeneous, pink-staining cylinders, or the tubule is filled with disintegrating hydropic cells. The epithelium of the loops of Henle in the outer cortex is similarly necrotic but in the inner portion there is less actual necrosis. Here the cells are markedly swollen and granular with many large vacuoles and pyknotic nuclei. The tubules contain an albuminous precipitate. The epithelium of the collecting tubules is also degenerated. The lumen of almost all the tubules is completely occluded by necrotic cell masses or by enormously swollen, degenerated, and desquamated cells. In the medulla hyaline casts are quite abundant and many tubules contain blood. There are a few irregular patches of interstitial edema in the inner cortex.

*Atoxyl.*—Following a small dose of atoxyl (Dog F), the only distinctive pathological changes consist in parenchymatous and fatty degeneration of tubular epithelium and slight congestion and hemorrhage in the boundary zone. The epithelium of the convoluted tubules stains palely, the cells are ragged and granular with irregular vacuolization, as shown in Fig. 8. There is some fragmentation of nuclei and many tubules contain albuminous precipitate. With a larger dose of atoxyl, however, (Dog E) a greater variety of changes is encountered. The vessels throughout are dilated and congested and there is some escape of blood into the interstitial tissue and tubules of the cortex. There is an extensive hemorrhage into the medulla, obscuring much of its structure. The glomeruli are swollen, but the glomerular tufts are irregular in size, some filling the capsular space while others occupy only a portion of it. Most of the tuft capillaries are dilated and filled with blood, and there is an albuminous precipitate in the capsular spaces. The tubular epithelium shows a peculiar series of changes. In general, it stains poorly; the cells of the convoluted tubules are swollen, granular,

ragged, and very hydropic; many have desquamated, choking the lumen of the tubule (Fig. 9). There is great irregularity in the preservation of the nuclei; many are fragmented or pyknotic, others are entirely gone. An occasional mitotic figure is seen. Practically all the tubules contain albuminous precipitate and there are a few hyaline casts. In the inner portion of the cortex there is an extreme degree of irregularity in the tubules, so that it is difficult to identify with certainty the various types. In this region the loops of Henle show a striking change; they are markedly enlarged, the cells are widely separated from one another, and are swollen and hyaline with pyknotic nuclei as shown in Fig. 9. Some of these cells are necrotic and desquamated. Another prominent feature of the atoxyl kidney is a profuse exudation of polymorphonuclear leucocytes which is most marked in the inner half of the cortex and the boundary zone.

An increase in the length of survival of the animal (Dogs C and D) gives an opportunity for the development of still further pathological changes in the kidney. In such cases, the hemorrhage invades the inner cortex as well as the medulla (Fig. 11). The tubular epithelium shows more marked disintegration of cells, many of which are hyaline while others are hydropic and swollen. There is an increase in the exudate in the tubules which consists of albuminous precipitate, colloid droplets, red blood cells, leucocytes, and casts, causing, in many instances, a marked compression of the tubular epithelium (Fig. 10). The number of casts is distinctly greater in these dogs of longer survival. In the medulla the tubular epithelium is almost completely desquamated, and the cells are intensely hyaline with pyknotic nuclei. There is a marked exudate between the tubules consisting of serum, fibrin, red blood cells, and a few leucocytes; no interstitial structures can be distinguished. Numerous mitotic figures are present in the epithelium of both cortex and medulla. Moreover, there is a very marked increase in the interstitial leucocytic exudate in these dogs of longer survival (Fig. 11).

#### DISCUSSION.

As far as we are able to determine from a pathological study of various arsenic kidneys, the idea that arsenical compounds as a class

produce vascular, in contradistinction to tubular, injury must be modified.

It is quite true that with a certain group of these compounds vascular lesions predominate, causing red kidneys, of which arsenious acid is the type. Even within this group of arsenicals, however, we are able to recognize certain differences in the character of the action of the different compounds. For instance, with salvarsan and neosalvarsan, hemorrhage tends to be restricted to the outer cortex or the boundary zone; the formation of hyaline thrombi in the glomerular capillaries is more pronounced; and there is a profuse albuminous exudate in the capsular space with desquamation of epithelial cells. Interstitial edema and degeneration and necrosis of the tubular epithelium are distinctly more pronounced than with arsenious acid.

As we study kidney lesions produced by other arsenicals, however, we find, as with arsacetin, that the predominating change is one of degeneration and necrosis of tubular epithelium. These pale kidneys offer a striking contrast to the red kidneys of vascular injury. Moreover, other pale kidneys, such as those produced by such substances as arsenophenylglycine and atoxyl, in which the tubular injury is the predominant feature, may show well marked vascular injury. In these cases, the hemorrhage may be zonal in character, involving much of the medulla or even the lower portion of the cortex. The tuft capillaries may be dilated and congested with more or less albuminous precipitate in the capsular space, and there may be an exudation of serum, fibrin, red blood cells, and leucocytes into the interstitial tissues.

The histological changes in the kidneys produced by a particular arsenical compound, while elastic, are quite characteristic of the action of the compound and accord with the gross appearance of the organ. Therefore, in surveying this series of arsenic kidneys from a microscopic as well as from a gross pathological point of view, we are able to differentiate two extreme types; *i. e.*, the red and the pale. The red kidney is essentially one of vascular injury, the pale kidney is predominantly one of tubular necrosis. In addition, various transitional or subgroups exist, in which the kidney, although belonging to the red type, shows relatively a great degree of

tubular injury and *vice versa*. In regard to the tubular necrosis in these kidneys, the prompt and active regeneration of the tubular epithelium following the injection of such compounds as arsacetin, arsenophenylglycine, and atoxyl, seems to preclude the possibility that the wide-spread tubular necrosis produced by these substances can be regarded as a purely anemic phenomenon. Hence, we cannot ascribe to arsenical compounds, as a class, the property of producing a purely vascular nephritis; but we must recognize the fact that arsenical compounds produce characteristic renal lesions which may be either predominantly vascular or tubular in type and that the mode of action and the character of the lesions produced are bound up with the chemical constitution of the compound.

#### SUMMARY.

1. We have shown that the type of renal lesion produced by compounds of arsenic varies widely: while some arsenicals produce changes in which vascular injury predominates, others produce an equally dominant tubular injury.

2. In either of these groups the character and degree of the vascular or tubular injury produced by different compounds shows further variation, such that the lesions of different arsenicals of the same group are not identical. Each compound of arsenic that we have tested, therefore, produces a lesion-complex in the kidney that is relatively characteristic for that compound.

3. The mode and character of the action of arsenicals are dependent upon the chemical constitution of the compound.

#### EXPLANATION OF PLATES.

The illustrations are all from untouched photomicrographs. Magnification,  $\times 208$ .

#### PLATE 61.

FIG. 1. Arsenious acid. Dog B. Section from the outer cortex. There is marked dilatation and congestion of vessels, including the glomerular capillaries, with hemorrhage into the interstitial tissues. The tubular epithelium is swollen and degenerated.

FIG. 2. Galyi. Dog S. Section from the outer cortex. The vessels are congested and there is a slight interstitial hemorrhage. The tuft capillaries are uni-

formly dilated and contain blood and there is a slight albuminous precipitate in the capsular space. Tubular degeneration and disintegration are marked.

PLATE 62.

FIG. 3. Salvarsan. Dog Q. Section from the outer cortex. Dilatation and congestion of vessels with slight hemorrhage. The glomeruli are swollen and the tuft capillaries contain blood and numerous hyaline thrombi. There is an albuminous precipitate in the capsular space. Tubular epithelium shows degeneration and slight necrosis.

FIG. 4. Salvarsan. Dog Q. Section from the inner cortex. Marked interstitial edema. Partial disintegration of glomerular tuft and accumulation of epithelial cells and cell detritus in the capsular space. Degeneration and necrosis of tubular epithelium.

PLATE 63.

FIG. 5. Neosalvarsan. Dog R. Section from the outer cortex. The glomeruli are swollen; the tuft is compressed; the glomerular capillaries are congested and contain numerous hyaline thrombi. There is an abundant albuminous precipitate in the capsular space. Many tubules show a massive necrosis, others show degeneration and disintegration of epithelial cells with pyknotic nuclei.

PLATE 64.

FIG. 6. Arsacetin. Dog I. Section from the midcortex. Extensive necrosis *en masse* of the epithelium of the convoluted tubules with marked degeneration and slight necrosis of the loops of Henle. Vessels and glomeruli normal.

PLATE 65.

FIG. 7. Arsenophenyglycine. Dog L. Section from the outer cortex. The glomeruli are large, the tufts are compressed, and the glomerular vessels are partially collapsed. The capsular epithelium is swollen and desquamated and the capsular space is filled with necrotic cellular debris. Degeneration and necrosis of tubular epithelium are marked.

PLATE 66.

FIG. 8. Atoxyl. Dog F. Section from the outer cortex. The vessels and glomeruli are normal, except for a slight albuminous precipitate in the capsular space. The epithelium of the convoluted tubules is extremely ragged and degenerated with slight necrosis.

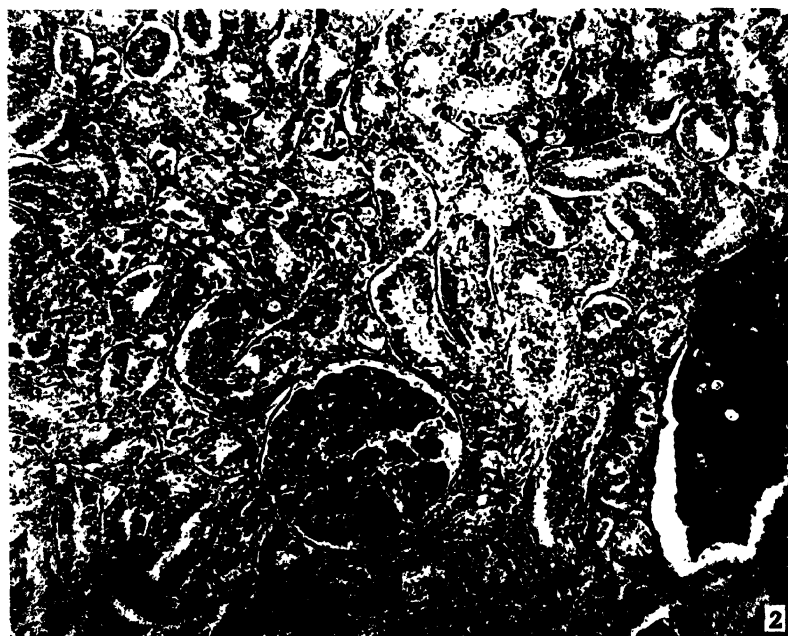
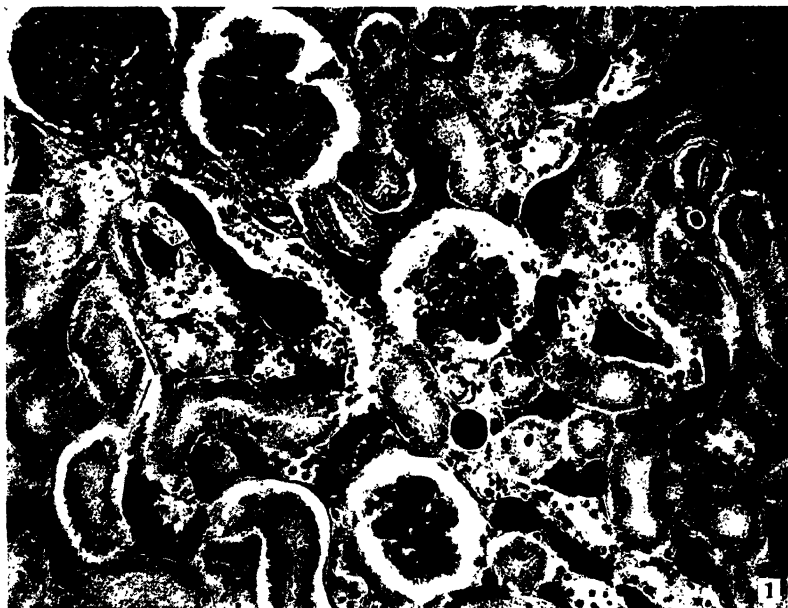
FIG. 9. Atoxyl. Dog E. Section from the midcortex. The glomeruli are slightly enlarged, the capillaries of the tuft are slightly congested, and the capsular space contains an albuminous precipitate. The epithelium of the convoluted tubules is swollen, granular, and hydropic, and in places shows necrosis. The loops of Henle are markedly dilated; the cells are hyaline with pyknotic nuclei and are widely separated from one another. Many cells are desquamated. There is a diffuse hemorrhage and polymorphonuclear exudate into the interstitial tissues and occasionally into the tubules.



## PLATE 67.

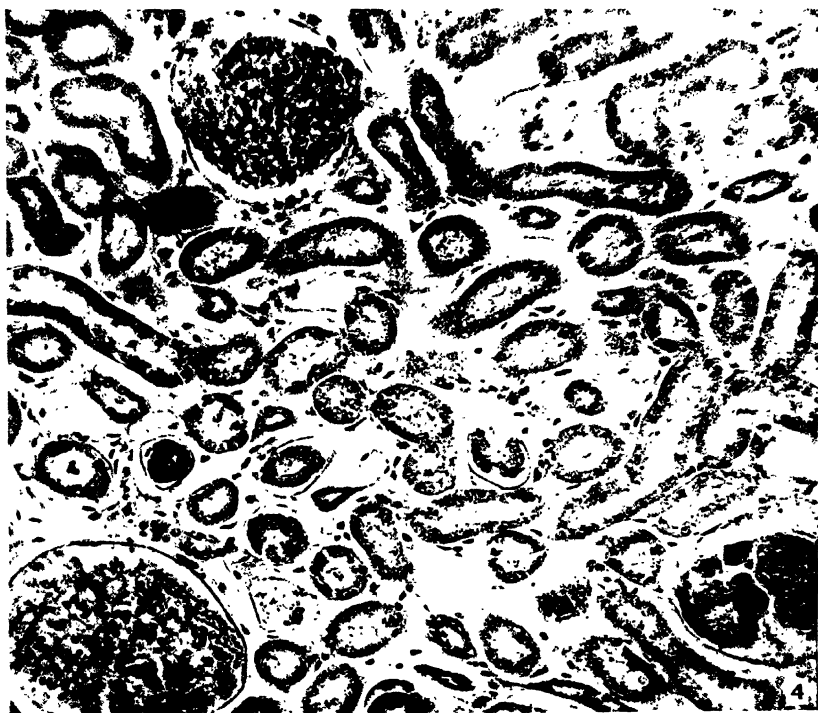
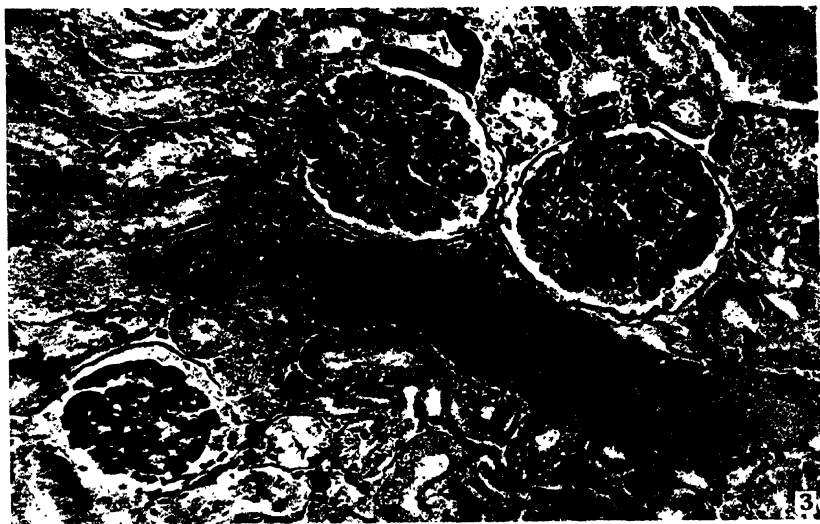
FIG. 10. Atoxyl. Dog D. Section from the outer cortex. The glomeruli are enlarged. The tuft is slightly compressed and the capsular space contains an abundant albuminous precipitate. The epithelium of the convoluted tubules is extremely ragged and hydropic and in some areas the cells are necrotic and desquamated. Other tubules are filled with a granular precipitate, hyaline droplets, and an occasional cast, and the epithelium of these tubules is compressed.

FIG. 11. Atoxyl. Dog D. Section from the inner cortex. The glomeruli are as in Fig. 10. The tubules throughout are necrotic, and filled with cellular detritus or granular casts. There is a marked interstitial hemorrhage with an exudate of polymorphonuclear leucocytes.



(Pearce and Brown: Changes in Arsenic Kidneys.)









(Pearce and Brown: Changes in Arsenic Kidneys.)

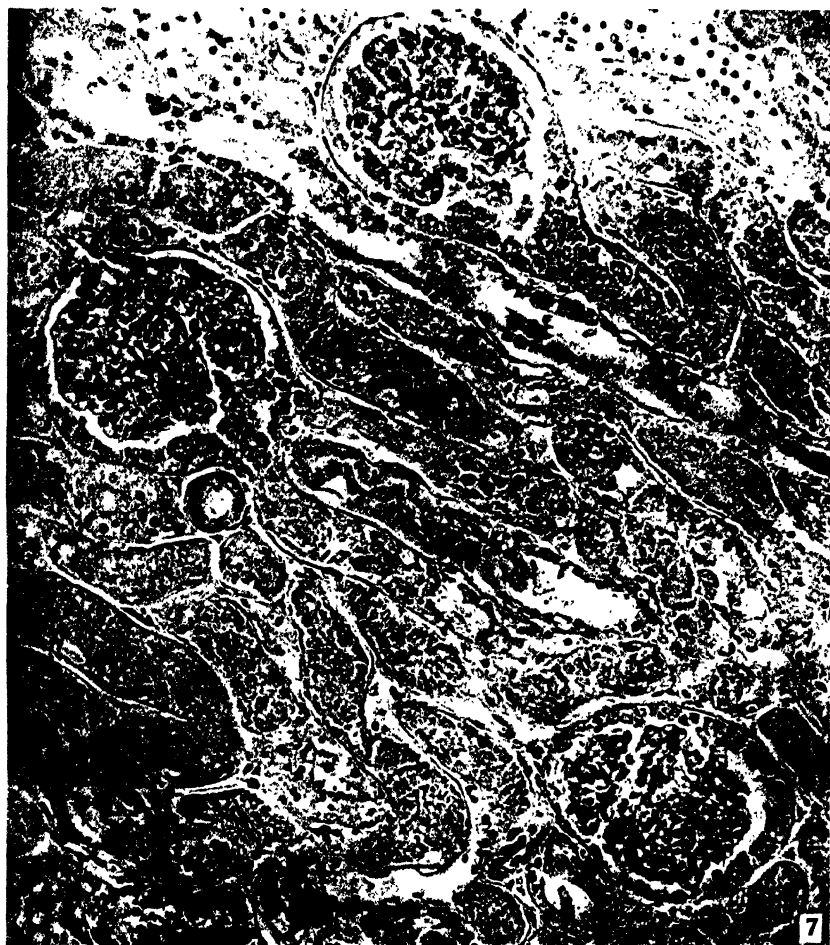




(Pearce and Brown: Changes in Arsenic Kidneys.)

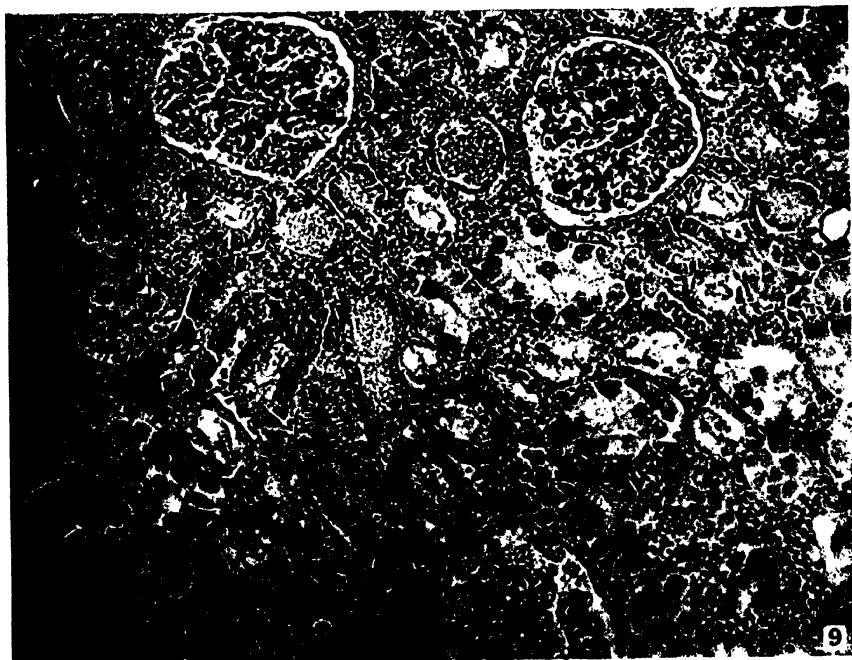






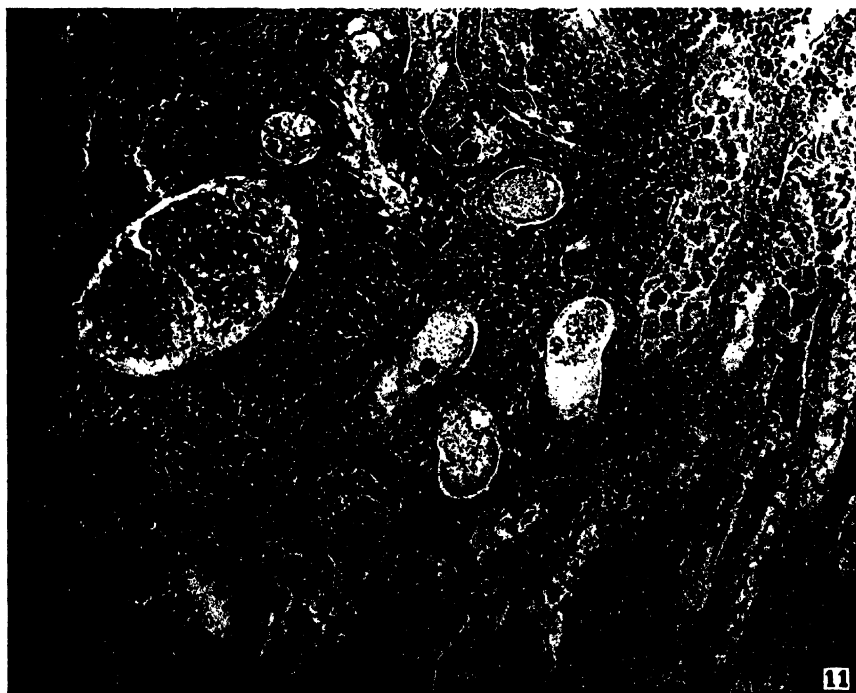
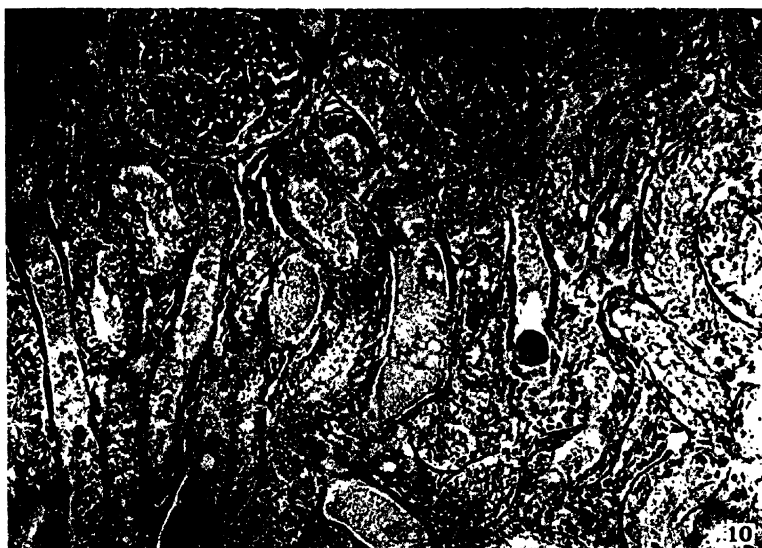
(Pearce and Brown: Changes in Arsenic Kidneys.)





(Pearce and Brown: Changes in Arsenic Kidneys.)





(Pearce and Brown: Changes in Arsenic Kidneys.)



## CHEMOPATHOLOGICAL STUDIES WITH COMPOUNDS OF ARSENIC.

### III. ON THE PATHOLOGICAL ACTION OF ARSENICALS ON THE ADRENALS.

By WADE H. BROWN, M.D., AND LOUISE PEARCE, M.D.

*(From the Laboratories of The Rockefeller Institute for Medical Research.)*

PLATES 68 TO 73.

(Received for publication, June 24, 1915.)

In spite of the unusual interest that has been manifested in both the ductless glands and experimental arsenic therapy during recent years, the action of arsenicals upon the adrenals, which is one of the most constant and perhaps important features of arsenical intoxication, has remained practically unnoted.

This phase of arsenical action was first observed by us in the course of our chemotherapeutic studies. The adrenals were so constantly involved in the toxic action of our series of arsenicals that we were led to suspect that the tendency to adrenal injury was not peculiar to our compounds but was shared, to a greater or less degree, by other arsenicals. We instituted a series of experiments, therefore, to determine whether such substances as arsenious and arsenic acids, sodium cacodylate, atoxyl, arsacetin, arsenophenylglycine, salvarsan, and neosalvarsan possessed a similar tendency to adrenal injury. These substances were chosen as representative and accessible types of organic and inorganic compounds of trivalent and pentavalent arsenic.

#### EXPERIMENTAL.

##### *Materials.*

The routine tests of the above named drugs were made upon male guinea pigs weighing 400 to 500 grams, though supplementary evi-



dence was obtained from both rabbits and dogs which were used in order to facilitate intravenous administration of the drugs.

### *Technique.*

Sterile solutions of the substances were injected intraperitoneally. The arsenious and arsenic acids were dissolved in the theoretical amount of sodium hydroxide and given in a 1 per cent solution. The solutions of salvarsan and neosalvarsan were prepared as for clinical use. All the other compounds were given in as dilute solution as possible, but the concentration necessarily varied somewhat with the amount of the drug given.

### *Dose.*

On account of a lack of accurate information as to the toxic action of some of the arsenicals, we were forced to employ a wide range of doses before obtaining results that could be regarded as comparable. The following figures (Table I) were found to represent roughly the doses in grams per kilo of body weight causing death in guinea pigs in one to three days, except in the instance of arsacetin and salvarsan, where the largest doses used are stated, though they did not kill.

TABLE I.

Drug.	Dose per kilo of body weight. gm.
Arsenious acid .....	0.010 to 0.012
Arsenic acid .....	0.015 " 0.020
Sodium cacodylate (Merck) .....	0.700 " 1.000
Atoxyl .....	0.080 " 0.100
Arsacetin .....	0.300
Arsenophenylglycine .....	0.250 (or less)
Salvarsan .....	0.150
" rabbit (intravenous) .....	0.100 to 0.150
Neosalvarsan .....	0.300 " 0.350

The effects of three types of intoxication were studied with most of the compounds; *i. e.*, the effect of an acutely fatal dose, the effect of single large, sublethal doses, and the effect of repeated large doses.

Gross evidences of injury were noted, and with each animal the right adrenal was preserved in Zenker's fluid; the anterior half of the left adrenal in Müller's fluid, to preserve the chromaffin; and

the posterior half in 10 per cent formalin. Sections were always taken from corresponding levels. The Zenker and Müller material was sectioned in paraffin and stained with hematoxylin and eosin. Frozen sections were made from the formalin-fixed tissue and stained for lipoids with Herxheimer's Scharlach R counterstained with aqueous alum hematoxylin.

As the prime object of the experiments was to determine a single fact,—the presence or absence of adrenal injury as a result of arsenical intoxication—the scope of the experiments does not justify a detailed description of the action of each compound. The description given is a composite one based on the results obtained with the various arsenicals upon the adrenals of the guinea pig. Since all the compounds do not affect the adrenals in precisely the same manner, however, such peculiarities in their action as we have noted may be briefly indicated.

### *The Normal Adrenal of the Guinea Pig.*

To facilitate description and as a basis for comparison, we wish to refer briefly to certain features of the normal adrenal of the guinea pig. The adrenals of adult male guinea pigs of the weights used show certain differences that accord in general with differences in the color of the animals. The cortex is grossly divisible into two zones: an outer waxy zone and an inner pigmented zone. In black guinea pigs the adrenals are relatively large with a narrow rim of waxy cortex sharply demarcated from a broad, intensely pigmented, inner zone. In white guinea pigs the other extreme exists; namely, small adrenals with a relatively broad rim of waxy cortex which gradually merges with a relatively narrow and slightly pigmented inner zone. These markings are doubly important since they furnish landmarks and standards for gross pathological changes in the adrenal cortex and correspond quite accurately with the normal distribution of lipoids (Figs. 1 and 2).

It so happens that the microscopic as well as the gross changes produced by arsenicals conform so closely to these divisions of the cortex that they can be most conveniently described upon such a basis of division. Without raising the question, therefore, of the relation of these zones to the generally recognized microscopic divi-

sions, the glomerulosa, the fasciculata, and the reticularis, we have used the designations, Zones 1, 2, and 3. Zone 1 corresponds with the zona glomerulosa; Zone 2 with the waxy or lipoid-containing fasciculata; and Zone 3 with the pigmented portion of the cortex which is also easily recognizable with the microscope on account of the difference in the character of the cells of Zones 2 and 3.

### *Gross Pathological Changes.*

Large doses of all the arsenicals tested cause an acute swelling of the adrenals, usually accompanied by congestion of the surface vessels with scattered foci and streaks of hemorrhage. On section the organs are very soft; the waxy cortex appears slightly gray and translucent and may be streaked with red. Frequently a distinct red line separates the waxy and pigmented zones. The latter area is usually very soft and depressed, but rarely shows any early change in its extent or the intensity of its pigmentation. The medulla is apt to be poorly defined, soft, and somewhat congested.

Later (after forty-eight to seventy-two hours) the adrenals, though still swollen, are quite pale. On section the organ is firmer, and the waxy zone of the cortex is usually distinctly widened, gray, and translucent. The pigmented cortex is narrowed and the intensity of pigmentation is decreased. The original line of contact between these two zones is indicated by a conspicuous narrow band of opaque yellow, gray, or pink tissue. The medulla is usually normal in appearance or somewhat more conspicuous than normally.

### *Microscopic Changes.*

*Lipoids.*—We have observed two types of alteration in the lipid content of the adrenal: change in the size of the droplets and change in the amount and distribution of the lipoids. Very early, large droplets of lipid material appear among the fine granules normally present in the cells of Zone 2 of the cortex. These large droplets are most numerous at the outer and inner edges of the zone and in the latter location, the junction of Zones 2 and 3, there appears to be an actual increase in the amount of lipid present (Fig. 3).

Subsequently lipid material increases in Zone 1 and begins to accumulate in considerable amounts in Zone 3 (Fig. 3), while at the

same time the amount of lipoid diminishes in Zone 2; this decrease is first apparent in the middle and inner half of the zone (Figs. 3, 4, and 5). Finally, a general depletion sets in and the lipoids disappear completely from the inner half of Zone 2, then from Zones 1 and 3, leaving only scattered droplets in the outer half of Zone 2 and possibly fine granules at the line of separation of Zones 2 and 3 (Figs. 4, 5, and 6).

Very rarely have we been able to demonstrate lipoid material in true medullary cells. In a few instances, fine granules have been observed in these cells.

*Vascular Changes.*—In the early stages of arsenical poisoning, the vessels of both the cortex and medulla of the adrenal are dilated and filled with blood. Hemorrhage is marked with some compounds (arsenious and arsenic acids and sodium cacodylate) especially in Zone 2 of the cortex, at the junction of Zones 2 and 3, and between the cortex and medulla (Fig. 7). Hyaline or leucocytic thrombi are occasionally seen in the vascular spaces and a slight leucocytic exudate and interstitial edema are frequently present.

*Cellular and Structural Alterations.*—The cortical cells of the adrenal show a variety of degenerative changes. The cells of Zone 1 are usually swollen; their cytoplasm is granular and slightly vacuolated. The cells of Zone 2 suffer most: the sharp outlines and spongy cytoplasm of these cells are soon lost; they become irregular, ragged, granular, and vacuolated; some are enormously swollen with pale-staining cytoplasm while others are shrunken. Hyaline cells are seen here and there and necrotic cells are numerous in extreme cases. All these cellular changes are again most pronounced at the junction of Zones 2 and 3 (Fig. 8). In Zone 3, cells that are usually finely granular show some small vacuoles and a coarsely granular or hyaline cytoplasm. Necrotic cells in this zone are most numerous at its inner edge.

The architecture of the cortex is frequently disturbed. The cell columns may be broken up and the cells appear completely isolated or in irregular clumps and columns.

The cells of the medulla also show evidence of injury. They are frequently ragged with scant, pale-staining, granular cytoplasm and shrunken, pyknotic nuclei. The most striking feature of the

change, with some compounds, is the presence of large numbers of colloid droplets within and among the cells of the medulla (Fig. 9). Necrotic cells may be numerous.

*Chromaffin.*—All the arsenicals that we have tested exercise some influence upon the chromaffin content of the adrenal. Some compounds (arsenious and arsenic acids) seem to cause but a slight reduction in the chromaffin content as judged by the color of the medullary cells of tissue fixed in Müller's fluid. Judged by the same standards, other arsenicals produce an extreme depletion of the chromaffin (sodium cacodylate, salvarsan, and neosalvarsan) as shown by comparing Figs. 10, 11, and 12.

*Recovery.*—The injury phase of the action of arsenicals upon the adrenals develops with a varying rapidity and persists for a variable length of time; with some compounds the changes develop rapidly and recovery is equally rapid, while with others the reverse is true. Regeneration of cortical cells is usually rapid and mitotic figures may be seen as early as twenty-four hours after the administration of the drug but are not numerous until forty-eight to seventy-two hours (Fig. 13). Here activity is most marked in the outer half of Zone 2. Six to twelve mitotic figures frequently occur in a single high power field of the microscope. Mitotic division occurs to a limited degree in the cells of Zones 1 and 3. The cells of the medulla also regenerate by mitotic division (Fig. 14). We observed mitotic figures in these cells in several instances after sodium cacodylate, atoxyl, and arsenophenylglycine, but they were never numerous.

Perhaps as a sequel to the injury produced in the medulla, round-celled or polyblastic infiltration is of frequent occurrence and is usually accompanied by some fibroblasts and an increase in endothelial cells (Figs. 15 and 16). To a less degree changes similar to those in Fig. 16 have been seen in control animals, which leaves some doubt as to the significance of this particular type of infiltration.

#### DISCUSSION.

Thus far, all the compounds of arsenic that we have tested have exhibited a definite action upon the adrenals of the guinea pig, but it by no means follows that the character and degree of such action

are identical for all arsenicals. On the contrary, while certain features of the action appear to be common to a number of compounds, other features of the action may be quite distinctive. Thus, arsenious acid and sodium cacodylate show a strong tendency to produce congestion and hemorrhages in the adrenals, while such compounds as arsenophenylglycine and arsacotin produce most marked disturbances in the lipoid content, with but slight tendency to congestion or hemorrhage. In like manner, arsenious acid seems to cause relatively slight alteration in the chromaffin content, while sodium cacodylate acts strongly on the medullary cells.

We must also recognize the fact that the relative importance of adrenal injury in the lesion-complex is another variable. With some arsenicals the effect upon the adrenals may be distinctly overshadowed by injury to other organs, while with other compounds the injury inflicted upon the adrenals plays a prominent part in the toxic manifestations.

We may conclude, therefore, that some factor other than the mere presence of arsenic must exercise a distinct influence upon the activity of these compounds; namely, their chemical constitution.

Finally we must differentiate between the action of toxic doses and the action of therapeutic doses of arsenicals upon the adrenals. Since the action of toxic doses indicates a strong selective affinity of the adrenals for compounds of arsenic, we may legitimately infer that with therapeutic doses injury might give place to stimulation, a conception which clinical experience with some arsenicals seems to justify.

However, generalizations as to the action of arsenicals on the adrenals should be made with caution. From these experiments concerning the action of compounds of arsenic upon the adrenals, we believe that a wide field of investigation has been opened up and that future work will justify our belief in the importance of the action of arsenicals upon the adrenals.

#### SUMMARY.

1. Toxic doses of all arsenicals of which we have any knowledge produce definite pathological changes in the adrenals of guinea pigs. These changes include congestion, hemorrhage, disturbances in the

lipoid content, cellular degenerations and necroses, and reduction in the chromaffin content.

2. The character and severity of the injury produced by different arsenicals varies with the chemical constitution of the compounds.

3. From these facts, we believe that adrenal injury is an important factor in arsenical intoxication and suggest that therapeutic doses of some arsenicals may produce adrenal stimulation.

### EXPLANATION OF PLATES.

The illustrations are all from untouched photomicrographs. Those showing lipoids are from frozen sections stained with Herxheimer's Scharlach R counter-stained with aqueous alum hematoxylin. Figures showing chromaffin are from tissue fixed in Müller's fluid and stained with hematoxylin and eosin. All doses of drugs are expressed in gm. per kilo of body weight.

#### *Changes in the Lipoids of the Adrenal Cortex.*

##### PLATE 68.

FIG. 1. Lipoids of normal adrenal cortex. Black male guinea pig. The lipid, which appears as black granules, is sharply confined to the relatively narrow outer rim of the cortex.  $\times 80$ .

FIG. 2. Lipoid of normal adrenal cortex. White male guinea pig. The lipid extends over more than one-half of the cortex and is irregularly demarcated at the inner edge of the zone.  $\times 80$ .

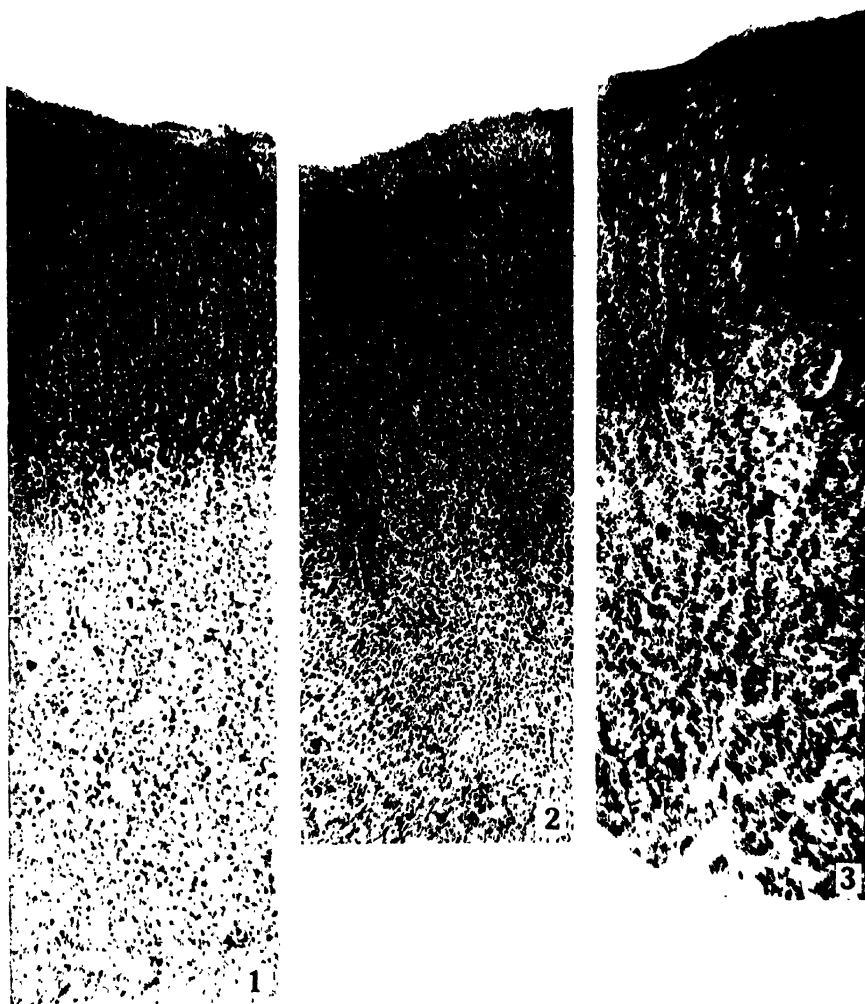
FIG. 3. Increase in the amount of demonstrable lipid and an abnormal distribution produced by arsenophenylglycine 0.250 gm. Animal killed after 48 hours. The lipid is distributed over the entire cortex and is especially abundant at the junction of Zones 2 and 3. The photographic intensity of the lipid in this figure had to be considerably suppressed in the original photomicrograph in order to preserve some detail.  $\times 80$ .

##### PLATE 69.

FIG. 4. A later stage of the same type of change as that in Fig. 3. A distinct decrease of the lipoids is here apparent in the inner half of Zone 2. 2 doses of arsenophenylglycine 0.100 gm., followed on the 5th day by arsenophenylglycine 0.050 gm. Animal died in 24 hours. No postmortem decomposition.  $\times 80$ .

FIG. 5. Still more pronounced change in the amount, distribution, and character of the lipid droplets. Neosalvarsan 0.300 gm. Guinea pig killed after 48 hours.  $\times 80$ .

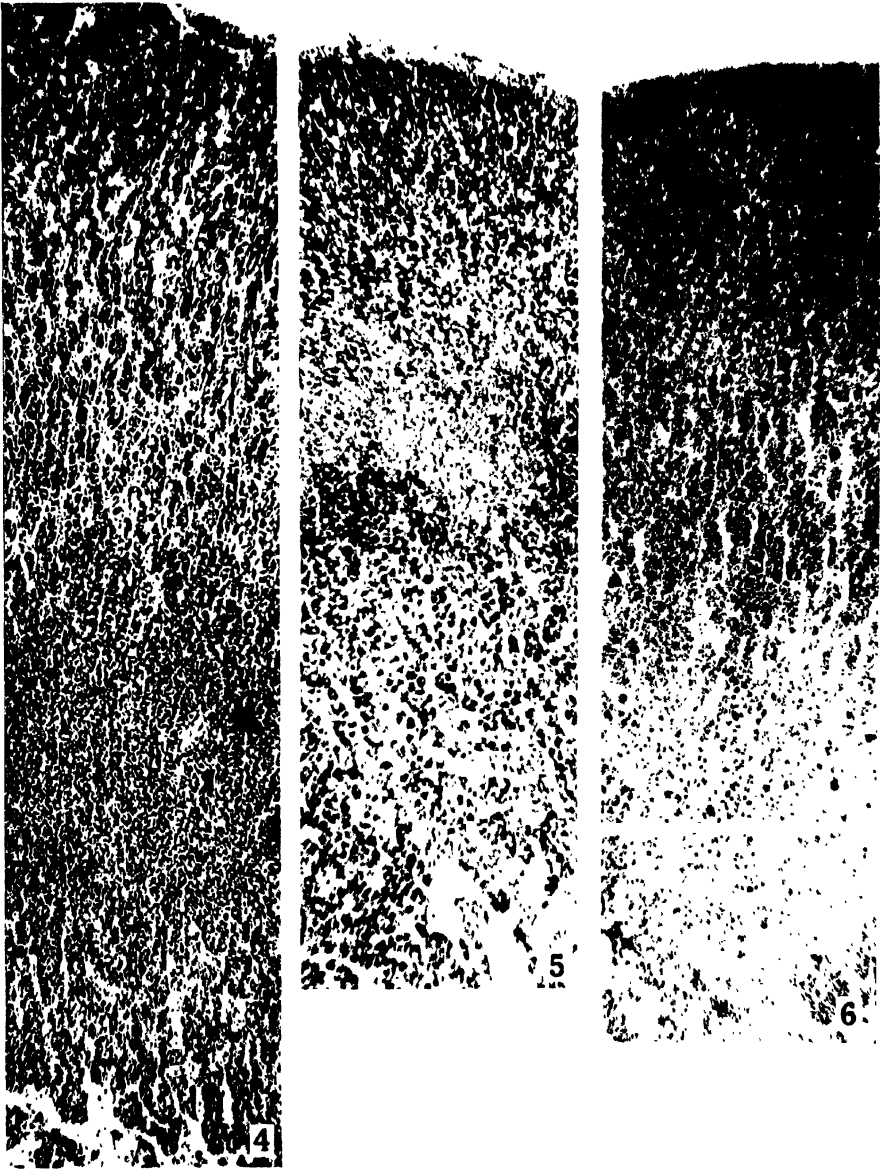
FIG. 6. Extreme depletion of lipoids produced by 4 doses of arsacetin (0.100, 0.200, 0.200, 0.200 gm.) within 16 days. Guinea pig killed 3 days after the last dose. The lipid is mostly in the form of large droplets with some very fine granules in the cells through the middle of the cortex.  $\times 80$ .



(Brown and Pearce: Action of Arsenicals on Adrenals.)

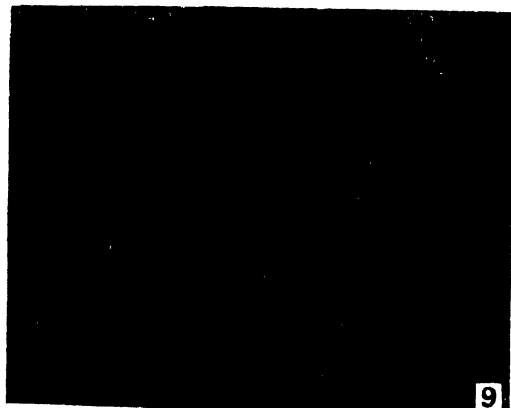
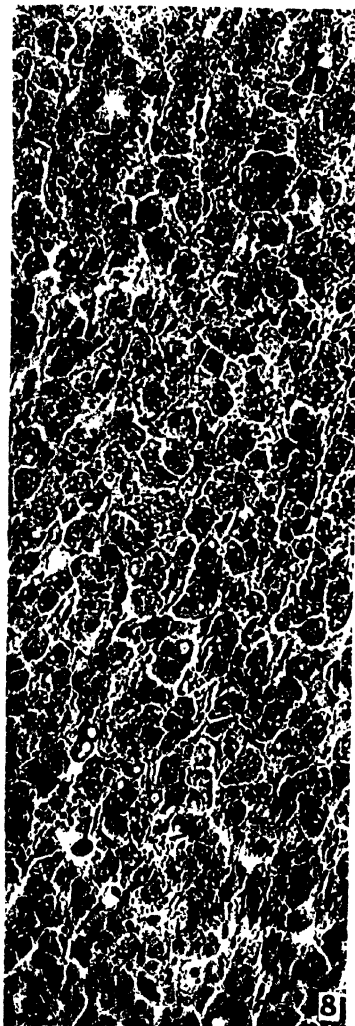
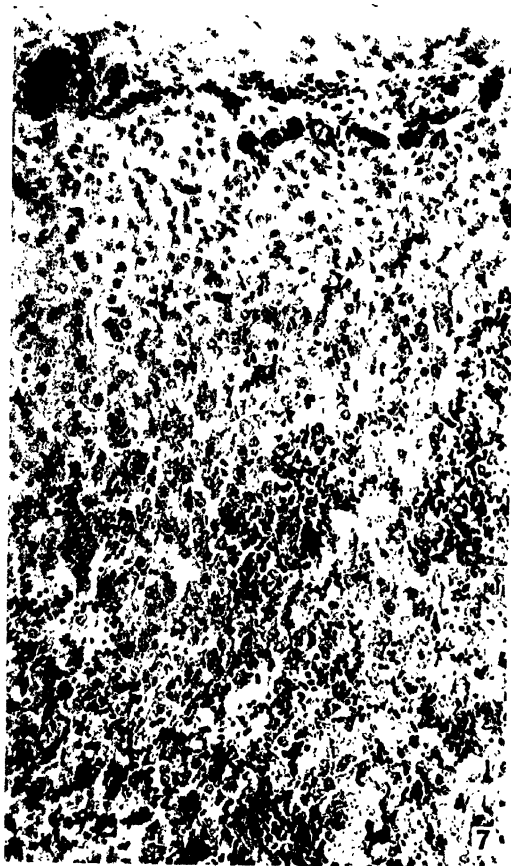






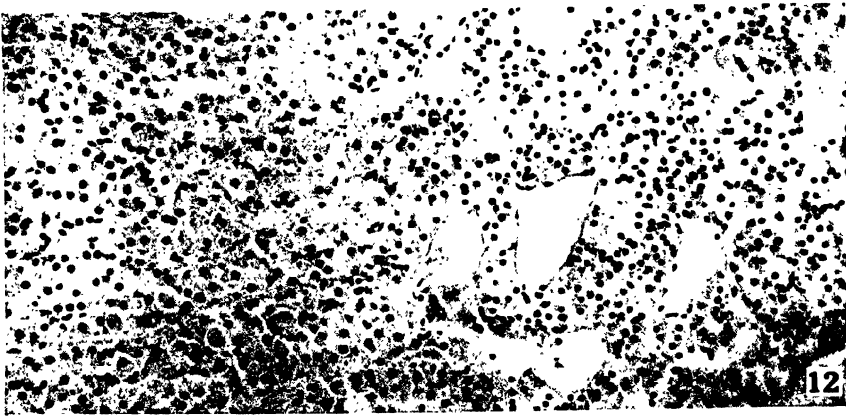
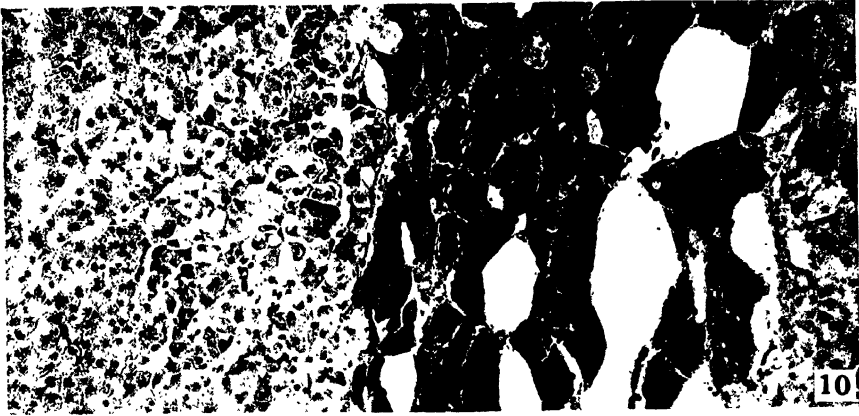
(Brown and Pearce: Action of Arsenicals on Adrenals.)





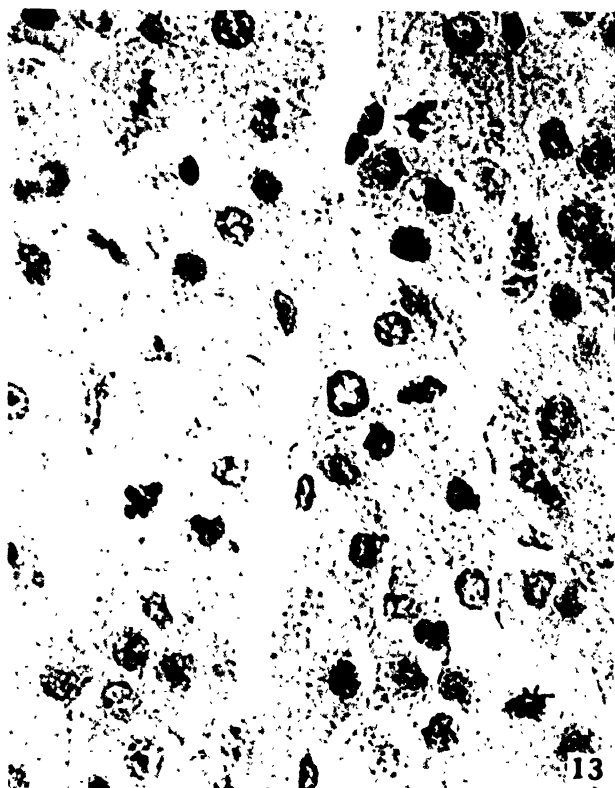
(Brown and Pearce: Action of Arsenicals on Adrenals.)





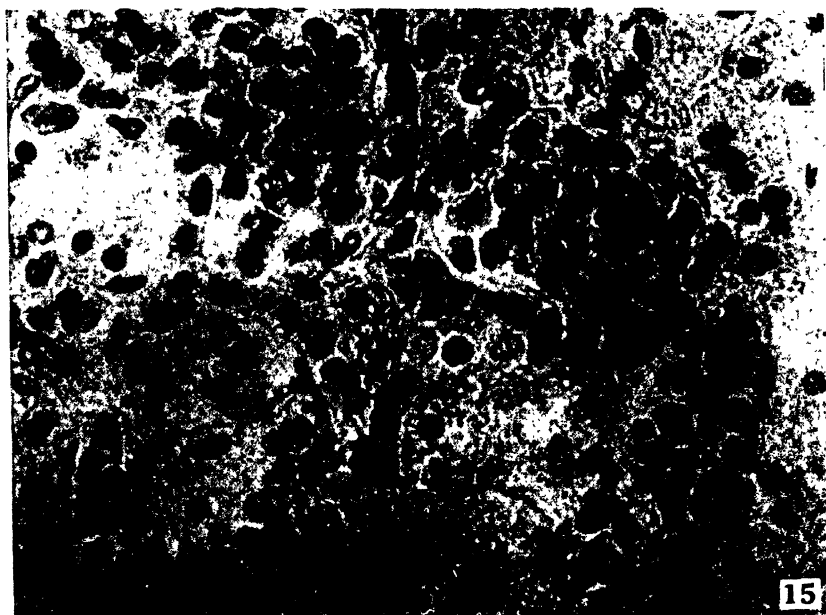
(Brown and Pearce: Action of Arsenicals on Adrenals.)











(Brown and Pearce: Action of Arsenicals on Adrenals.)



*Congestion, Hemorrhage, and Degeneration.*

## PLATE 70.

FIG. 7. Congestion and hemorrhage in the cortex of the adrenal produced by 2 doses of sodium cacodylate 0.700 gm. in 4 days. Guinea pig died on the 5th day. Hemorrhage is most marked in the inner half of Zone 2. The cortical cells also show degeneration and vacuolization.  $\times 200$ .

FIG. 8. Degeneration of the cells of the midzone of the adrenal cortex with slight leucocytic infiltration resulting from arsenophenylglycine. Same adrenal as in Fig. 4.  $\times 200$ .

FIG. 9. Colloid degeneration of medulla of the adrenal. Same animal as in Figs. 4 and 8.  $\times 775$ .

*Changes in the Chromaffin.*

## PLATE 71.

FIG. 10. Chromaffin of adrenal of normal guinea pig.  $\times 200$ .

FIG. 11. Reduction in the chromaffin content of the medulla with shrinkage and degeneration of medullary cells. Sodium cacodylate 0.700 gm. Guinea pig killed after 48 hours.  $\times 200$ .

FIG. 12. Reduction of chromaffin after 2 doses of neosalvarsan (0.190 gm. and 0.500 gm.) on the 5th day. Died within 18 hours. No postmortem decomposition.  $\times 200$ .

*Regeneration of Adrenal Cells.*

## PLATE 72.

FIG. 13. Cortical regeneration. Neosalvarsan 0.300 gm. Killed after 48 hours.  $\times 700$ .

FIG. 14. Mitosis in cell of medulla. Atoxyl, 3 doses of 0.050 gm. in 5 days. Guinea pig killed 3 days after last dose.  $\times 600$ .

*Infiltration in the Medulla.*

## PLATE 73.

FIG. 15. Infiltration of polymorphonuclear leucocytes and polyblasts into a degenerated area of the medulla. Arsenophenylglycine. Same adrenal as in Figs. 4, 8, and 9.  $\times 700$ .

FIG. 16. Infiltration of lymphocytes and fibroblasts in the medulla of the adrenal. Arsenious acid 0.005 gm. Killed after 3 days.

## A NOTE ON THE IMMEDIATE EFFECTS OF REDUCTION OF KIDNEY SUBSTANCE.<sup>1</sup>

By H. T. KARSNER, M.D., H. A. BUNKER, JR., A.B., AND  
G. P. GRABFIELD, M.D.

(From the Department of Pathology (Phillips Fund) of the Harvard Medical  
School, Boston.)

(Received for publication, June 11, 1915.)

Several investigators have studied the nitrogenous metabolism of animals following reduction of kidney substance; notably, Tuffier,<sup>2</sup> Bradford,<sup>3</sup> Bainbridge and Beddard,<sup>4</sup> Pearce,<sup>5</sup> and Pilcher.<sup>6</sup> Studies of nitrogen balance and partition have been made and somewhat contradictory results obtained. de Paoli<sup>7</sup> observed that large quantities of kidney substance could be removed without endangering life and that the minimum necessary for life is one-half of one kidney, or one-quarter of the total kidney substance. Tuffier, on the other hand, stated that life is possible with only 1.5 gm. of kidney substance per kilo of body weight (the total is about 7 gm. per kilo in the dog). Bradford found the danger limit reached at 2 gm. per kilo; this was confirmed by Pearce and by Pilcher.

As regards function Tuffier, as quoted by Bradford, states: "It is possible to remove quantities of kidney substance equal in weight and in volume to those of the sum of the two kidneys in such a way as yet to leave behind a considerable amount of kidney substance, as shown by postmortem examination; that this diminution in the amount of kidney substance is *not* accompanied by any functional disturbance of the urine, and that the urine and urea, after oscillations due to the operation, return to their normal amount."

Bradford found that excision of less than two-thirds of the kidney volume

<sup>1</sup> Aided by a grant from The Rockefeller Institute for Medical Research.

<sup>2</sup> Tuffier, T., *Études expérimentales sur la chirurgie du rein*, Paris, 1889; cited by Bradford, J. R., *The Results Following Partial Nephrectomy and the Influence of the Kidney on Metabolism*, *Jour. Physiol.*, 1898-99, xxiii, 415.

<sup>3</sup> Bradford, J. R., *loc. cit.*

<sup>4</sup> Bainbridge, F. A., and Beddard, A. P., *The Relation of the Kidneys to Metabolism*, *Proc. Roy. Soc., London, Series B*, 1907, lxxix, 75.

<sup>5</sup> Pearce, R. M., *The Influence of the Reduction of Kidney Substance upon Nitrogenous Metabolism*, *Jour. Exper. Med.*, 1908, x, 632.

<sup>6</sup> Pilcher, J. D., *On the Excretion of Nitrogen Subsequent to Ligation of Successive Branches of the Renal Arteries*, *Jour. Biol. Chem.*, 1913, xiv, 389.

<sup>7</sup> de Paoli, E., *Centralbl. f. Chir.*, 1892, xix, 78; cited by Bradford, *loc. cit.*

resulted in temporary increase of the watery part of the urine, which became lasting when two-thirds were removed; removal of "approximately three-quarters of the total kidney weight is followed by a very great increase in the amount of urinary water, and also by an increase in the amount of urea excreted." He found also a considerable increase in the nitrogenous extractives of the blood and tissues, particularly muscles, most marked after excision of three-quarters, but quite apparent after excision of two-thirds of the kidney substance. This excess was distributed just as after double nephrectomy and after the intravenous injection of urea; he states that the quantity in the muscles is too great to be accounted for by the mere products of normal metabolism.

Bainbridge and Beddard working on cats found that removal of approximately three-fourths of the total kidney substance is not constantly followed by an increased output of nitrogen, and that it takes place only in cats which have lost 22 per cent or more of their initial body weight at the time of its onset. They concluded further that the kidneys have no direct influence upon nitrogenous metabolism and that the increased output of nitrogen is simply the result of inanition. They found the cats still able to pass a concentrated urine and that the amount of urine is not necessarily increased beyond the normal.

Pearce, using dogs, confirmed the general results of Bainbridge and Beddard and concluded further that the metabolism condition of starvation is apparently the result of the gastro-intestinal disturbance constantly associated with extensive kidney reduction and not of a disturbance of general nitrogenous metabolism. He found that the gastro-intestinal disturbance was not due to diminished absorption and found no evidence to support a theory of internal secretion on the part of the kidney.

Pilcher, using both cats and dogs, reduced the kidney substance by ligation of branches of the renal arteries. He states that with but one-fourth of the kidney substance functioning the quantity of urine was practically normal; there is marked temporary prostration, anorexia, loss of weight, and increased nitrogen output with finally a return to normal, with a slight tendency to nitrogen retention.

#### REPORTS OF EXPERIMENTS.

Our experiments have been conducted because of the availability of the Folin methods<sup>8</sup> for the determination of the non-protein nitrogen of the blood.

Dogs were kept on a constant diet and determinations were made of the total non-protein nitrogen of the blood, obtained by aseptic heart puncture. The urine was collected in the ordinary metabolism cages and the total non-protein nitrogen and urea nitrogen were determined. All analyses were made in duplicate or triplicate.

<sup>8</sup> Folin, O., and Denis, W., Protein Metabolism from the Standpoint of Blood and Tissue Analysis, *Jour. Biol. Chem.*, 1912, xi, 161; New Methods for the Determination of Total Non-Protein Nitrogen, Urea and Ammonia in Blood, *ibid.*, p. 527.

All the blood results are reported in mg. per 100 cc. of blood. All the urine results are reported in mg. per twenty-four hours.

Five dogs were used, but the results can be summarized in the protocols of three (Tables I, II, and III).

TABLE I.

*Dog I.*

*Female, Weight 6,500 Gm. Urine Negative for Albumin. Diet 200 Gm. of Dog Biscuit.*

Date.	Blood. Total non-protein nitrogen	Urine.		
		Total non-protein nitrogen.	Urea nitrogen.	Amount.
Apr. 29 .....	36	1,840	—	66.
" 30 .....	44	2,520	1,861	100
May 1 .....	45	2,010	1,903	63
" 2 .....	Left kidney (weight 17 gm.) removed under ether anesthesia			
" 3 .....	44	1,192	859	201
" 4 .....	Slight diarrhea			
" 4 .....	43	1,451	1,357	159
" 7 .....	Diarrhea very severe			
" 7 .....	51	—	—	41
" 9 .....	44	—	—	—
" 24 .....	Diarrhea finally cleared up. Experiment resumed			
" 24 .....	38	—	—	—

At 12.30 p. m. the right kidney (weight 22 gm.) was removed and the animal bled at more frequent intervals, as follows:

		Total non-protein nitrogen in blood.
May 24.....	12.30 p. m.....Operation	
" 25.....	7.30 p. m.....	44
" 25.....	1.00 a. m.....	56
" 25.....	1.00 p. m.....	85
" 25.....	7.00 p. m.....	111
" 25.....	11.30 p. m.....	133
" 26.....	2.00 p. m.....	196
" 26.....	11.00 p. m.....	227
" 27.....	10.00 a. m.....	285
" 27.....	2.00 p. m.....Animal found dead.	

Autopsy shows slight superficial infection of the abdominal wound; the abdominal cavity is clean; a few cc. of gray, slightly turbid fluid are found in the left pleural cavity.

TABLE II.

Dog 2.

Female, Weight 6,000 Gm. Urine Negative for Albumin. Diet  $2\frac{1}{2}$  Dog Biscuits.

Date.	Blood. Total non-protein nitrogen.	Urine.		
		Total non-protein nitrogen.	Urea nitrogen.	Amount.
Aug. 7.....	24	—	—	cc. —
" 8.....	18	4,005	3,337	89
" 10.....	—	2,278	1,960	245
" 11.....	Left kidney (weight 35 gm.) was removed under ether anesthesia			
" 12.....	43	—	—	0
" 13.....	27	2,156	1,705	55
" 14.....	26	3,149	2,350	94
" 15.....	21	3,393	3,237	390
" 16.....	—	2,470	2,041	130
" 18.....	Right kidney was removed under ether anesthesia			

Total non-protein nitrogen  
in blood.

Aug. 18.....	11.45 a. m.....	Operation	
	11.45 a. m.....		31
	11.45 p. m.....		72
Aug. 19.....	11.45 a. m.....		95
	11.45 p. m.....		114
" 20.....	11.45 a. m.....		143
	6.00 p. m.....		154
	12.00 midnight.....		165
" 21.....	12.00 noon.....		208
	10.00 p. m.....		225
" 22.....	9.00 a. m.....		282
	5.00 p. m.....	Found dead.	

Autopsy showed no sepsis or other abnormalities.



TABLE III.

*Dog 3.**Female, Weight 4,000 Gm. Urine Negative for Albumin. Diet 120 Gm. Chopped Beef and 2 Dog Biscuits.*

Date.	Blood. Total non-protein nitrogen.	Urine.		
		Total non-protein nitrogen.	Urea nitrogen.	Amount.
Aug. 24.....	23	1,823	1,568	cc. 49
" 25.....	—	3,725	3,053	192
" 28.....	Upper pole of right kidney (weight 6 gm.) was removed under ether anesthesia through lumbar incision by Dr. W. C. Quinby			
" 29.....	26	4,480	3,360	224
" 30.....	20	4,480	4,032	224
" 31.....	22	3,800	2,800	200
Sept. 1.....	Entire left kidney (weight 22 gm.) was removed under ether anesthesia			
" 2.....	28	—	—	150
" 3.....	41	4,620	3,135	330
" 4.....	35	3,995	2,820	235
" 8.....	41	Diar- rhea		
" 11.....	33			
" 12.....	26			
" 14.....	33			
" 18.....	Remainder of right kidney (weight 17 gm.) was removed under ether anesthesia			

Total non-protein nitrogen  
in blood.

Sept. 18.....	3.00 p. m.....	Operation	
	3.00 p. m.....		35
" 19.....	12.00 noon.....		68
" 20.....	11.30 a. m.....		152
	5.00 p. m.....		172
" 21.....	9.30 a. m.....		227

2.00 p. m. Death occurred as the blood was being withdrawn.

The last operation wound was infected; otherwise the autopsy showed nothing abnormal.

## DISCUSSION OF RESULTS.

From these data it may be said that removal of approximately one-sixth of the kidney substance (Dog III) results in no marked alteration of the non-protein nitrogen of the blood or of the urine, although there is, as Pilcher expresses it, a slight tendency to accumulation<sup>9</sup> in the blood and increased output in the urine for a

<sup>9</sup> The term accumulation is used instead of retention to avoid confusion with the use of the latter term as expressing the holding of nitrogen in the body for metabolic and constructive purposes.

period of twenty-four (blood) or forty-eight hours (urine), probably to be explained by the operation. Excision of one-half the total kidney substance in the case of Dog II was followed by a distinct accumulation in the blood during the first twenty-four hours, associated, however, with anuria; such accumulation, however, did not appear in the corresponding period in Dog I, which was not anuric. As seen in both dogs, however, the output for the first forty-eight hours appears to be diminished, thus again showing a slight tendency toward accumulation for at the most forty-eight hours. The removal of approximately two-thirds of the kidney substance (Dog III) was followed by a very slight increase in output, and after twenty-four hours a slight but distinct increase in the non-protein nitrogen of the blood which lasted for three days.

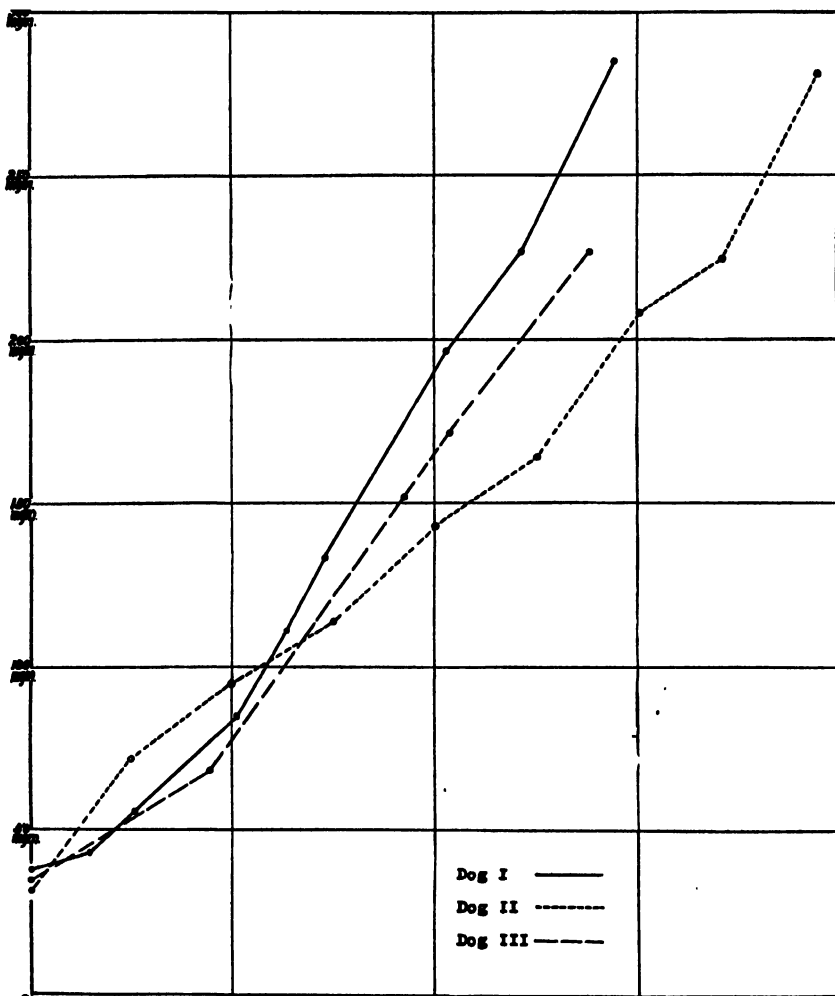
An examination of the amount of urine and its concentration shows that reduction of the kidney substance down to approximately one-third does not prevent that fragment from excreting urine in normal amounts and concentrations.

Diarrhea was found to occur frequently and followed the excision of only one-half the kidney substance. None of the dogs lost markedly in weight, and this reduction in no case reached the critical 22 per cent of Bainbridge and Beddard.

The results following the complete removal of kidney substance are shown graphically in Text-fig. 1. In Dogs I and III the rate of increase was considerably less in the first twenty-four hours than in the succeeding periods. On the second and third days the rate of increase was practically the same and from rough calculations based on the output for twenty-four hours was about what it should be per 100 cc. of blood for a twenty-four hour period. It is easily possible that operative shock might account for the relatively slight increase in the first twenty-four hours.

In Dog II the results are more constant, although in the last twenty-four hours there was a slight exacerbation of rate. In other words, for this animal the rate of increase per 100 cc. of blood should be roughly 57 mg. per day per 100 cc., so that the amounts would be for each succeeding day 88, 145, 203, and 260, whereas they actually were 95, 143, 208, and 282 (allowing twenty-one and one-half hours for the last day). It is regretted that the

actual intake of nitrogen following the total nephrectomy was not estimated and the results here presented are regarded as suggestive



TEXT-FIG. 1. Chart showing total non-protein nitrogen in blood following complete nephrectomy.

rather than conclusive. Further studies are being carried out in an attempt to elaborate on and elucidate this increase in total non-protein nitrogen in relation to intake, distribution in the body, and other factors which are known to influence protein metabolism.

## EXPERIMENTAL ARTHRITIS IN THE RABBIT. A CONTRIBUTION TO THE PATHOGENY OF ARTHRITIS IN RHEUMATIC FEVER.

By HAROLD KNIEST FABER, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, July 16, 1915.)

The significance of experimental arthritis in the rabbit has for many years been a matter of dispute. This may be attributed to the lack of data bearing upon the mechanism involved in the production of the lesions. It may be of interest to scrutinize the literature for facts which may relate to the conditions under which arthritis is, or fails to be, produced.

The first to produce arthritis in animals with pure cultures of streptococci was Loeffler (1), who published his results in 1884. Making cultural examinations of the exudate in the throats of patients with scarlet fever and diphtheria he isolated, besides the bacillus which now bears his name, several strains of streptococci, or micrococci, as he called them. Two of these strains, one from a fatal case of scarlet fever, the other from a fatal case of diphtheria, produced suppurative arthritis upon intravenous inoculation in 9 out of 12 rabbits. With Fehleisen's original strain of *Streptococcus erysipelatis* and by the same method he then produced a similar arthritis in 2 out of 3 rabbits. Smears from the pus in all cases showed abundant organisms and cultures made from 13 of them showed growth in 4, or 30 per cent—a high figure, considering the relatively crude methods employed. Most of the rabbits died.

Attention is called to the following points in Loeffler's work. There was between the time of injection and the development of arthritis an incubation period of 4 to 8 days, during which the animals were apparently normal. Arthritis followed only those injections which were given intravenously. Subcutaneous injections were followed by local infection usually ending in death without the evolution of joint lesions. The cultures, as shown by the mortality, were highly virulent. The infecting organism could be easily demonstrated in smears from the joint exudate and in a considerable percentage the cultures were also positive.

The streptococcus isolated by Wassermann (2) gave similar results, though it was probably not so virulent. The results are not reported in detail.

The diplococcus of Poynton and Paine (3) produced arthritis in 4 out of 9 rabbits injected (original report). The incubation period was 2 to 4 days. Two of the affected rabbits died on the 10th and 20th days, respectively, and two were killed. Only one recovered. Smears and cultures from the joint exudate were constantly positive. Here again the strain was evidently highly virulent.

Meyer (4) cultivated streptococci from tonsillar crypts of patients with rheumatic fever and produced arthritis in rabbits with these strains. The incubation period was 6 to 8 days and it was usually possible to demonstrate the organisms in numbers by smear and culture. Protocols and details are lacking.

Cole (5) in 1904 made a more careful study of experimental streptococcal arthritis. Using several strains isolated from widely varying conditions he produced with all a definite arthritis in rabbits,—also by intravenous inoculation. The organisms could usually be easily demonstrated in the joint exudate by smear and culture.

The most careful study of the one injection arthritis produced with virulent streptococci was made by Jackson (6). Using a highly virulent streptococcus isolated from the milk epidemic in Chicago she studied the joint reaction histologically at different periods after the injection. This report shows that 2 hours after injection streptococci could be found in the vessels of the periarticular tissues, that at 10 hours intravascular collections of leucocytes were present, while at 24 hours exudation and migration of leucocytes into the joint cavity had occurred. There is then a refractory period following the arrival of organisms at the joint during which no evident inflammatory reaction occurs, and this we may call the incubation period. It must be inferred that this reaction depends for its promptness and severity upon the virulence of the infecting organism. This is in accordance with the experiments of Dreyer (7) who found that the degree of reaction after injection of organisms into the joint of the rabbit is a good measure of their virulence. The point of most interest to us in Cole's paper is, however, the fact that two of his rabbits, inoculated with a less virulent strain, developed arthritis only after a second injection. In one of these rabbits smears and cultures were negative from all the affected joints except one and in this both smear and culture were positive. In several of the other animals spontaneous recovery occurred and in these Cole was able to provoke a relapse by another intravenous injection of the same organism. One attempt to cause a relapse with another strain of streptococcus failed.

Shaw (8) in 1904 and Schloss and Foster (9) in 1913 found that in monkeys arthritis occurred only after the second intravenous injection. Rothschild and Thalheimer (10) in 1913 note in their protocols that several of their rabbits with arthritis following intravenous inoculation of *Streptococcus mitis*, a slightly virulent organism, received five and six injections. In about one-third of these, cultures from the joint were positive.

It appears, then, that according to the virulence of the streptococcus used and possibly also according to the variety, two types of reaction may occur after intravenous inoculation. In one type, produced by streptococci of high virulence, the joint is attacked after the first injection. It is to be noted, however, that even in these instances a period of incubation is always observed. In this type of reaction cultures and smears from the affected joint are usually positive and the organisms are present in considerable numbers. In the second

type of reaction for which less virulent streptococci are used, no demonstrable joint lesion follows the first injection but a very definite arthritis does follow the second or, as will be shown, a still later injection. In other words the development of arthritis in these cases is conditioned probably upon some anterior process set up in the affected joint. In this type, cultures and smears from the joint frequently show no bacteria and, if bacteria are found, they are in much smaller numbers than in instances of the first type.

The number of definitely controlled and carefully reported experiments bearing upon the second type of reaction is quite small. A larger series is reported below.

In 1913 a Belgian investigator, Herry (11), reported a series of experiments which indicated the existence of a third type of reaction. In a remarkably large number of cases of rheumatic fever he was able to isolate a streptococcus from the blood or articular exudate. From this organism he obtained by a process of extraction with normal saline, desiccation, trituration, reextraction, and centrifugalization, an endotoxin soluble in water. By injection of this into the joint followed by an intravenous injection of the living organism 8 to 15 days later he was able to produce constantly in rabbits a definite arthritis in the joint originally treated. Cultures and smears from the joint were positive for about a week after the intravenous injection. Those experiments throw a clearer light upon the process of joint localization, but unfortunately they suffer from incompleteness.<sup>1</sup>

#### EXPERIMENTAL.

Experiments were therefore instituted with the object of treating joints with streptococci so that they might be more reactive to later intravenously injected streptococci and with the hope that arthritis might be made to localize in the joint so treated. It was thought that if this could be done the mechanism of joint localization might be partly explained.

<sup>1</sup> I have attempted without success to repeat Herry's experiments with extracts of the *S. mitis* used for the other experiments in this paper. Herry's method calls for the use of the supernatant fluid of a bacterial suspension after centrifugalization and it is probable that a certain number of organisms remained in this material. However, the author states that the same results may be obtained with endotoxin passed through a Chamberland filter. It should be stated that the subject of endotoxins in streptococci has been thoroughly studied and that present day opinion denies their existence. Herry's experiments are, however, illuminating.

A strain of *Streptococcus mitis seu viridans* was used for most of the experiments which was kindly lent by Dr. E. Libman of Mt. Sinai Hospital, New York. It was isolated by him from the blood of a patient with subacute endocarditis. It does not hemolyze blood, and grows on blood agar in small, discrete, dry, grayish colonies. It clouds glucose-ascitic agar. In broth it clumps and sinks to the bottom of the tube, leaving the medium clear. It is insoluble in rabbit bile and has no capsule. Since coming into our hands its pathogenicity for rabbits has been slight, a single intravenous inoculation of the contents of two 12 ounce Blake bottles of agar<sup>2</sup> failing to cause more than the slightest and most transient symptoms. This strain is designated No. 7. No. 4 is a similar organism from a similar source. No. 59 is a *Streptococcus viridans* obtained one year ago by Dr. Homer F. Swift from a case of pericarditis. It does not fall readily into any of the common groups of Andrewes and Horder. It is only slightly pathogenic for rabbits. Pn. I is a Type I pneumococcus isolated at the Hospital of The Rockefeller Institute. Pn. S. Afr. is a pneumococcus of Type IV isolated in South Africa from the Kaffir epidemic. The latter is not very pathogenic for rabbits. The *Bacillus typhosus* is a laboratory strain.

TABLE I.

*Rabbits Receiving One Intravenous Injection of Streptococcus 7.*

Rabbit No.	Amount injected.	Period of observation after injection.	Arthritis.	Remarks.
		<i>days</i>		
1	3 agar slants	49	0	Intra-arterial.
3	1 " slant	49	0	
5	$\frac{1}{2}$ Blake bottle	31	0	
6	$\frac{1}{4}$ " "	39	0	
7	" " "	13	0	
9	" " "	39	0	
19	1 agar slant	14	0	Slight limp on 16th day. No swelling or other evidence of arthritis.
38	" " "	37	0	

Total ..... 8 rabbits.

Arthritis ..... 0.

<sup>2</sup> The surface of the medium in the average Blake bottle is equal to that of 10 agar slant tubes.

Rabbits of medium size (of 1,200 to 1,800 grams' weight), usually females, were used. All injections were given in the ear vein except one or two into the femoral artery. The latter method was found to have no localizing effect in the corresponding leg.

Table I shows the effect of one injection, Table II of two injections, and Table III of three or more injections.

TABLE II.

*Rabbits Receiving Two Intravenous Injections of Streptococcus 7.*

Rabbit No.	Amount of 1st injection.	Interval between 1st and 2d injections.	Amount of 2d injection.	Arthritis.	Period of observation after 2d injection.
		<i>days</i>			<i>days</i>
8	$\frac{1}{4}$ Blake bottle	9	$\frac{1}{4}$ Blake bottle	0	45
36	$\frac{1}{4}$ agar slant	6	1 " "	0	20
K	" "	15	$\frac{1}{4}$ " "	0	1
L	" "	15	" " "	0	13

Total ..... 4 rabbits.

Arthritis ..... 0.

It appears that with the streptococci used two sensitizing doses were needed before any of the rabbits developed arthritis. Smears from the joint exudate in a few cases showed a few streptococci and in three cases the cultures were positive. Cultures were made in most of the cases by planting the fluid in tall tubes of glucose-ascitic agar and in the others in ascitic bouillon, both methods appearing to be equally efficacious.

The fluid in different rabbits varied from a thick, practically purulent exudate to one showing only a moderate opalescence. All the exudates examined, with the exception of that from Rabbit 17, were viscid. All contained numerous polymorphonuclear leucocytes, and some large mononuclear lymphocytes and large endothelial cells. The last named and occasionally the other two types of cell frequently contained inclusions which were interpreted as phagocytized cocci. They were Gram-negative and somewhat larger than the cocci injected. Unaltered cocci were rarely seen within the cells. It is interesting to note that similar inclusions were described by Bosc and Carrieu (12) and have been seen by the writer in cells in exudate from human rheumatic fever.



TABLE III.  
Rabbits Receiving Three or More Intravenous Injections of *Streptococci*.

Organism.	Rabbit No.	Amount of 1st injection.	Interval between 1st and 2d injections.	Amount of 2d injection.	Interval between 2d and 3d injections.	Amount of 3d injection.	Interval between 3d and 4th injections.	Amount of 4th injection.	No. of injections.	Arthritis.	Joint affected.	Remarks.
<i>Streptococcus</i>	7	1/4 Blake bottle	9 days	1/4 Blake bottle	63 days	1/4 Blake bottle	— days	—	3	+	Both knees	Positive culture from joint fluid.
"	15	" "	9	" "	63	" "	—	—	3	+	" "	Positive culture from joint fluid.
"	16	1/4 "	1	" "	1	1/4 "	33	1/4 Blake bottle	4	0	—	
"	17	" "	1	" "	1	" "	33	1/4 Blake bottle	4	+	Left wrist	Joint attacked 23 days after 3d injection. After recovery, a 4th injection caused a relapse.
"	24	" "	3	" "	1	" "	27	1/4 Blake bottle	4	0	—	
"	25	" "	3	" "	1	" "	27	1/4 Blake bottle	4	0	—	
"	J 59	1/10 Blake bottle each day for 8 days. 3 cc. broth culture	2	3 cc. broth culture	1	3 cc. broth culture	—	—	8	+	Both knees	
"	77	1 agar slant	1	1 agar slant	1	1 agar slant	—	—	3	0	—	
"	4	4 cc. broth culture	3	" "	1	" "	—	—	3	+	Right shoulder	Positive culture from joint fluid

Total rabbits injected with *Streptococcus* 7..... 4.  
 " " " 59..... 2. " " " 1.  
 " " " 4..... 1. " " " 0.

The next series of experiments was made in an attempt to sensitize a joint with streptococci so that arthritis in that joint would follow a later intravenous injection of the same organism.

The left knee was used in all cases. The injections were made by the following technique:

A suspension of the organism was made by scraping the surface of an agar growth into sterile salt solution. In a few cases a broth culture was employed. The suspension was drawn into a syringe and the needle inserted through the patellar ligament just below the patella. It was carefully pushed proximally, avoiding the bone surfaces as much as possible until it was felt to slip forward easily. This indicated that the point of the needle was in the synovial pocket under the quadriceps. The suspension was slowly injected and a little of it made to flow back into the syringe by pressure over the lower part of the quadriceps (piston test). This was returned to the joint and the needle quickly withdrawn.

After a few trials it was possible to inject the joint without infecting any of the periarticular tissues, thus giving a sharply localized reaction. This procedure caused an arthritis which usually subsided in 2 to 4 weeks, the joint and its contents returning to their normal state as far as could be determined microscopically. Dead bacteria were rapidly phagocyted, while the living resisted destruction and removal for a longer time. In all but a few cases dead bacteria were used for sensitization.

At a varying period after the inflammation and its products had been shown by examination of the synovial fluid to have disappeared an intravenous injection of the same organism was given. The results are given in Table IV.

The exudate in all cases except Rabbit 84 failed to show growth and recognizable streptococci were seen in the smears only once. The picture seen in the smears had the same characteristics as in the cases of successive intravenous inoculation without direct sensitization. In Rabbit 58 a second intravenous injection after the reaction following the first had subsided was also followed by a transient articular reaction.

The effect of similar procedure with other bacteria was then investigated with the results shown in Table V.

The condition of susceptibility in a joint to intravenous injections of streptococci has been referred to as one of sensitization, but it

TABLE IV.

*Rabbits Receiving an Intravenous Injection of Streptococcus 7 after Treatment of the Left Knee with the Same Organism.*

Rabbit No.	Amount injected into knee.	Interval.	Amount injected intravenously.	Arthritis (gross signs).	Joint fluid.	
					Culture.	Smear.
26	$\frac{1}{2}$ Blake bottle, living	days 53	2 cc. broth culture	+	o	
34	1 cc.,* killed	28	1.5 cc. broth culture	+	o	P.n.l. +++ Inclusions +
35	" " living	28	1.5 cc. broth culture	+	o	P.n.l. +++ Inclusions +
45	0.2 " "	15	1 agar slant	+	o	P.n.l. ++ A few cocci.
53	0.5 " killed	60	2 cc.	+	o	P.n.l. ++ No bacteria.
58	0.2 " "	35	$\frac{1}{2}$ agar slant	+	o	P.n.l. +++
58†		65	1 " "	+	o	P.n.l. ++ No bacteria.
61	" " "	19	" " "	+	o	P.n.l. ++ No bacteria.
65	10 mg. dried cocci, precipitated with alcohol	17	" " "	+	o	P.n.l. +
66	10 mg. dried cocci, precipitated with alcohol	31	2 cc.	+	o	P.n.l. ++
84	0.2 cc., killed	36	$\frac{1}{2}$ Blake bottle	o‡	+	No p.n.l. No inclusions.
111	" " "	14	" " "	+	o	P.n.l. ++++
112	" " "	14	" " "	±	o	Inclusions + P.n.l. ++
113	" " "	14	" " "	++	o	Inclusions + P.n.l. +++
114	" " "	14	" " "	+	o	Inclusions + P.n.l. +++
115	" " "	14	" " "	++	o	Inclusions + P.n.l. ++ Inclusions +

Total rabbits treated with Streptococcus 7..... 15. Arthritis..... 14.

\* Unless otherwise specified, figures given indicate amounts of a bacterial suspension consisting of the contents of a Blake bottle agar growth at 24 hrs. taken up in 10 cc. of normal saline. The total is equivalent to the growth on 10 agar slants.

† 65 days after the arthritis following the 1st intravenous injection had subsided a 2d intravenous injection was given and was likewise followed by marked inflammation of the joint.

§ 4 days later slight reddening and swelling of the joint appeared and polymorphonuclear leucocytes were found in the fluid, but the culture showed no growth.

TABLE V.

*Rabbits Receiving Intravenous Injections of Streptococcus 59, Bacillus typhosus, or Pn. S. Afr. after Treatment of the Left Knee with the Homologous Organism.*

Rabbit No.	Amount injected into knee.	Interval.	Amount injected intravenously.	Arthritis (gross signs).	Joint fluid.		Organism.
					Culture.	Smear.	
69	$\frac{1}{2}$ agar slant, killed	16 days	2 loops	0	—	—	<i>B. typhosus</i> .
91	0.5 cc., killed	29	$\frac{1}{2}$ agar slant	0	+	Some blood	" "
92	" " "	31	$\frac{1}{2}$ " "	0	0	No excess cells	" "
93	" " "	31	" " "	0	0	Normal, except that one cell shows inclusions	" "
95	" " "	29	" " "	0	0	Normal, except for one small clump of bacilli	" "
72	1 mg. bacteria sensitized by Gay's method	21	" " "	±	0	Normal	" "
73	1 mg. bacteria sensitized by Gay's method	29	1 " "	+	+	Cells increased, 80% p.n.l. No bacteria	S. 59
83	0.2 cc., killed	35	$\frac{1}{2}$ Blake bottle	0	0	P.n.l. ++ Inclusions +	" "
85	0.2 cc.	35	" " "	0	0	A few p.n.l. Inclusions +	" "
96	0.5 "	31	1 " "	0	0	A few p.n.l. Inclusions +	" "
97	" "	31	1 broth tube	0	0	Normal	Pn. S. Afr.
						A few p.n.l. No inclusions	" " "

Total rabbits treated with *B. typhosus* ..... 5. Arthritis ..... 0.  
 " " " " *Streptococcus 59* ..... 4. " ..... 2.  
 " " " " *Pn. S. Afr.* ..... 2. " ..... 0.

may be fairly asked whether any inflammation of the joint may not predispose to later joint affections. In other words, is the predisposing factor simply a non-specific inflammation or is it a specific sensitization? In order to answer this question a series of control experiments were made by injecting one sort of bacteria into the knee and following this with an intravenous injection of another sort (Table VI). By using two closely related strains of streptococci two doubtful (certainly very slight) reactions were obtained. By crossing with streptococcus and pneumococcus no reactions were obtained. It seems fair to conclude from these experiments that the reaction is due to a specific sensitization showing some evidence of a

TABLE VI.  
*Attempts at Cross-Sensitization.*

Rabbit No.	Inoculation into knee.		Inter-val.	Intravenous inoculation.		Ar-thrit-ia.	Joint fluid.		Remarks.
	Amount.	Organism.		Amount.	Organism.		Cul-ture.	Smear.	
52	0.5 cc. susp., killed by heat	Streptococcus 7	days 69	1/2 agar slant, living	Streptococcus 59	?	0	Moderate number cells, 90% p.n.l. No bacteria Normal	Slight or absent pal- pable swelling of knee.  No swelling.
67	10 mg. bacteria precipitated with alcohol	"	" 24	1/2 " "	Pn. II	0	0		
76	0.2 cc. susp., killed by heat	"	59 21	1/2 " "	Streptococcus 7	?	0	Moderate number cells, 90% p.n.l. No bacteria Rare	Very doubtful palpable swelling of knee.
83	0.2 cc. susp., killed by heat	"	7 35	1/2 Blake bottle, living	"	59 0	0	Few cells. No inclusions p.n.l. No inclusions	No swelling or other gross evidence of ar- thritis.
84	0.2 cc. susp., killed by heat	"	59 35	" " "	"	7 0	0	Normal. No p.n.l. No bacteria	No swelling or other gross evidence of ar- thritis.
106	0.3 cc. susp., killed by heat	Pn. S. Afr.	14	1/2 " "	"	" 0	0	Rare cells. 3 p.n.l. seen. No bacteria	No swelling or other gross evidence of ar- thritis.
107	0.3 cc. susp., killed by heat	" "	14	" " "	"	" 0	0	Rare cells. 1 p.n.l. seen. No bacteria	No swelling or other gross evidence of ar- thritis.
108	0.3 cc. susp., killed by heat	" "	14	" " "	"	" 0	0	No excess cells No bacteria	No swelling or other gross evidence of ar- thritis.
109	0.3 cc. susp., killed by heat	" "	14	" " "	"	" 0	0	Very few cells. No p.n.l. No bacteria	No swelling or other gross evidence of ar- thritis.
110	0.3 cc. susp., killed by heat	" "	14	" " "	"	" 0	0	A few cells. About 10% p.n.l. No bacteria	No swelling or other gross evidence of ar- thritis.

Total rabbits tested for heterologous sensitization ..... 10  
 " " showing joint reaction ..... 2 (doubtful).

group specificity comparable with the group agglutinations of certain bacteria.

*Pathological Anatomy.*

The arthritis following intravenous inoculation alone and that following intravenous inoculation after local sensitization presented the same gross and microscopic picture. An exception must be made in the case of Rabbits 14 and 15, in which a chronic arthritis with the continued presence of demonstrable living streptococci in the exudate followed the last injection. Here erosions in the articular cartilages were found. In the other rabbits the joint at autopsy showed the following changes: The synovial surface was moderately congested, the villi usually more so than the other parts. An excess of stringy fluid was usually present. The capsule was somewhat swollen. In cases which had reacted severely there was occasionally found after inflammation a small amount of inspissated exudate in the upper angle of the joint cavity. Sections showed a marked leucocytic infiltration of the villi and to a slighter degree of the subendothelial layer of the capsule. Examination of the adjacent cartilage and bone failed to show any noticeable change. Examination of the articular and periarticular tissues for bacteria by the Gram stain did not reveal the presence of organisms after a careful search. The vessels of the capsule and of the villi were moderately distended but no thrombi were seen. The reaction as seen under the microscope appeared to be mainly in the villi with the synovial membrane also playing a part.

DISCUSSION.

It is believed that the above experiments may throw some light upon the mechanism of acute arthritis. It seems to be established that the first attack of a highly virulent streptococcus can cause an arthritis and that the arthritis so produced is usually of a severe type with an exudate containing large numbers of viable organisms.

On the other hand, streptococci of lower virulence are frequently not able to produce arthritis at the first attack but at this time prepare the way for such an effect in a later attack.

It seems to be clearly proved that this preparatory or sensitizing process is, within narrow limits, a strictly specific one; *i. e.*, the organism used for the exciting, intravenous injection must be the same as that used for the sensitizing, intra-articular injection, else the reaction fails to occur.

It further appears that this preparation may be made by the introduction into the joint of the organism, living or dead, and it seems fair to conclude that the arthritis produced by successive intravenous injections results from a similar process; *i. e.*, a preliminary deposit of organisms in the joint leading to sensitization but not to gross inflammatory lesions, the latter resulting from a subsequent deposition of organisms. It is also shown that this reaction in a sensitized joint may be repeated several times in the same animal.

A close analogy between this reaction and the relapses in rheumatic fever can readily be drawn. Relapses are so common in this disease as to be one of its distinguishing characteristics. Thus in the figures given by Mosler and Valentin (13) 60 out of the 142 cases studied were suffering from their second or a later attack at the time of admission to the hospital. Cole produced 8 successive attacks, each followed by recovery, in a single rabbit.

Without assuming the specificity of any one organism the conception of relapsing arthritis as the effect of a virus upon an homologously sensitized joint may be fairly applied to the relapsing cases of human rheumatic fever.

The reaction of a sensitized joint in rabbits inoculated intravenously may properly be designated as an induced relapse and it will be seen from the protocol of Rabbit 17 that a second relapse can be induced by a second intravenous injection. From the ease and constancy with which lesions can be induced in sensitized joints by the method above outlined it is strongly suggested that the relapses, if not the primary arthritis, in rheumatic fever result from a virus reinvading the blood stream and provoking a reaction in a previously sensitized locus.<sup>3</sup>

<sup>3</sup> It may not be too wide a step to pass from the consideration of the effects induced in the joints to the phenomena observed in the endocardium and possibly even the myocardium in rheumatic fever. That they too are of relapsing nature is admitted; and that they also result from sensitization may be suggested.

In respect to the primary attack an analogy may be drawn from the fact above noted, that in rabbits and monkeys there is needed either a period of incubation, or, in most cases, a series of injections of the exciting organism before the joint is attacked. This fact suggests the hypothesis that some degree of sensitization, or of heightened activity on the part of the fixed cells is necessary before a definite and marked tissue reaction occurs.

It is not desired to state any opinion as to the identity of the organism causing rheumatic fever. In view of the fact that several different organisms can cause arthritis in the rabbit, that several different organisms can cause arthritis in man, and that the clinical manifestations of rheumatic fever vary widely it may well be that no one organism is constantly at fault. Nevertheless the streptococcus, and in most cases a member of the *viridans* group, has been the one most often found when cultures are positive. Further, this organism shows greater and more constant arthrotropic properties than any other now known. The preponderance of the evidence now available, therefore, is with the streptococcus. At this point the case must rest until further proof is offered.

#### SUMMARY.

By a process of sensitization described it was found possible to cause arthritis in rabbits constantly after one intravenous injection of the streptococcus.

This reaction is specific.

By intravenous inoculation, without previous sensitization, of the streptococcus used in these experiments it was possible to cause arthritis in rabbits only after three or more injections.

An analogy is suggested between the arthritis induced by sensitization and the relapses in human rheumatic fever.

A further analogy is suggested between the development in rabbits of arthritis after repeated intravenous injections and the development of the primary lesion in human rheumatic fever.

#### BIBLIOGRAPHY.

1. Loeffler, F., Untersuchungen über die Bedeutung der Mikroorganismen für die Entstehung der Diphtherie beim Menschen, bei der Taube und beim Kalbe, *Mitt. a. d. k. Gsndtsamte.*, 1884, ii, 421.



2. Westphal, Wassermann, and Malkoff, Ueber den infektiösen Charakter und den Zusammenhang von akutem Gelenkrheumatismus und Chorea, *Berl. klin. Wchnschr.*, 1899, xxxvi, 638.
3. Poynton, F. J., and Paine, A., The Etiology of Rheumatic Fever, *Lancet*, 1900, ii, 861.
4. Meyer, F., Zur Bakteriologie des akuten Gelenkrheumatismus, *Ztschr. f. klin. Med.*, 1902, xli, 311.
5. Cole, R. I., Experimental Streptococcus Arthritis in Relation to the Etiology of Acute Articular Rheumatism, *Jour. Infect. Dis.*, 1904, i, 714.
6. Jackson, L., Experimental Streptococcal Arthritis in Rabbits. A Second Study Dealing with Streptococci from the Milk Epidemic of Sore Throat in Chicago, 1911-12, *Jour. Infect. Dis.*, 1913, xii, 364.
7. Dreyer, L., Ueber Virulenzprüfung mittels intraartikularer Impfung, *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1912-13, lxvii, 106.
8. Shaw, W. V., Acute Rheumatic Fever and Its Etiology, *Jour. Path. and Bacteriol.*, 1904, ix, 158.
9. Schloss, O. M., and Foster, N. B., Experimental Streptococcic Arthritis in Monkeys, *Jour. Med. Research*, 1913-14, xxix, 9.
10. Rothschild, M. A., and Thalhimer, W., Experimental Arthritis in the Rabbit, Produced with *Streptococcus mitis*, *Jour. Exper. Med.*, 1914, xix, 444.
11. Herry, Contribution à l'étude du rhumatisme articulaire aigu; essai de pathogénie et de sérothérapie; étude clinique, anatomique, et expérimentale, *Bull. de l'Acad. roy. de méd. de Belgique*, 1914, xxviii, 76.
12. Bosc, F. J., and Carrieu, M., Inclusions intracellulaires dans le liquide articulaire du rhumatisme articulaire aigu, *Compt. rend. Soc. de biol.*, 1913, lxxiv, 1262.
13. Mosler, E., and Valentin, B., Zur Pathologie des akuten Gelenkrheumatismus, *Berl. klin. Wchnschr.*, 1910, xlvii, 1778.

## BACTERIOLOGICAL RESULTS IN CHRONIC LEUKÆMIA AND IN PSEUDOLEUKÆMIA.<sup>1</sup>

BY C. H. BUNTING AND J. L. YATES.

In a recent article Simon and Judd (Jour. Amer. Med. Asso., 1915, LXIV, 1630) report the cultivation of a diphtheroid organism similar to that found in Hodgkin's disease from the lymph-glands of a case of acute leukæmia, confirming the findings of Steele in a similar case (Boston Med. and Surg. Jour., 1914, CLXX, 123). Although the authors make no strong claim for the etiological significance of their finding, they suggest that the occurrence of the same organism in two cases must be more than fortuitous and emphasize the necessity of further investigations in this group of diseases.

It is with this in mind that we desire to report our bacteriological investigation of two recent cases. The first of these was a case of chronic lymphatic leukæmia of two years' duration, seen by the courtesy of Dr. A. W. Gray, of Milwaukee; and the second was a case of acute lymphosarcoma (or pseudoleukæmia) referred by the Jackson Clinic, of Madison, Wis.

Dr. Gray has kindly furnished the following clinical notes of the first case:

Jan. 18, 1913. W. H. M., male, white; traveling salesman; aged 40.

*Family History.*—Negative.

*Past History.*—Childhood diseases and frequent attacks of tonsillitis; tonsils always large. The laryngologist at the Mayo Clinic removed adenoids two years ago. The patient cannot remember whether or not the glands in the neck were enlarged at the time the tonsils were removed, in fact he had not noticed the enlargement until his attention was called to it during the examination.

*Present Condition.*—Cold in the head, deafness and aphonia.

*Examination.*—Fairly well nourished, weight 140 pounds. Heart and lungs

<sup>1</sup> This work has been aided by a grant from The Rockefeller Institute for Medical Research.

negative; spleen palpable; pharynx, fauces and vault of mouth show a peculiar red color which does not suggest acute inflammation; granular pharyngitis marked; fragments of tonsillar tissue present; faucial tonsil large; cervical, axillary and inguinal glands markedly enlarged, especially the anterior cervical, many of which were about the size of a hazel-nut. The blood examination showed the leucocyte count to be 186,000. A differential count was not made at this time. Hæmoglobin, 85% (Sahli).

The patient was under active treatment from February 1, 1913, when the leucocyte count was 175,200. He was given benzol in varying doses. Roentgen-ray radiation was given by Dr. O. H. Foerster. The leucocyte count, on March 29, 1913, had dropped to 8200. Sodium cacodylate, gr.  $\frac{3}{4}$ , hypodermically, was given at this time once in two days for two or three weeks. There was a decided

The following counts have been made in the first case:

Date.	W. b. c.	Neutr. %	Eos. %	Bas. %	S. Lymph. %	L. Mono. %	Trans. %
II-18-13.....	175,000	48.8	0.0	1.2	7.5	32.5	10.0
III-29-13.....	8,000	66.0	0.0	0.0	5.4	20.6	8.0
IV-17-13.....	6,200	94.5	1.0	0.0	2.0	1.5	1.0
V-24-13.....	6,200	40.0	1.0	0.0	56.5	2.0	0.5
VI- 3-13.....	6,200	41.5	0.5	0.0	48.5	8.0	1.5
VII-21-13.....	7,080	28.0	0.67	0.0	50.7	19.3	1.33
VIII-25-13.....	10,000	26.0	1.33	0.0	44.0	27.0	1.67
IX-22-13.....	10,100	20.0	1.5	0.0	35.0	40.0	3.5
X-13-13.....	15,000	23.0	3.0	0.0	43.0	29.0	2.0
XI-23-13.....	6,800	35.1	1.3	0.0	56.0	2.6	4.0
XI-25-13.....	8,800	14.5	0.4	0.0	82.3	1.0	1.8
IV-20-14.....	21,200	15.75	0.75	0.0	81.75	0.5	1.25
V- 7-14.....	15,600	16.0	3.0	0.0	74.0	1.0	6.0
VII- 7-14.....	13,100	15.0	0.5	0.0	81.5	2.5	0.5
IX-18-14.....	14,800	13.0	1.0	0.0	61.0	2.0	23.0
XII-30-14.....	25,500	5.5	0.5	0.0	90.0	0.5	3.5
I- 1-15.....	41,000	3.0	0.0	0.0	96.0	0.0	1.0
III- 2-15.....	63,200	3.5	0.0	0.0	55.0	41.5	0.0
III-31-15.....	92,900	.....	.....	.....	.....	.....	.....
IV-16-15.....	99,400	.....	.....	.....	.....	.....	.....
IV-26-15.....	161,200	.....	.....	.....	.....	.....	.....
V- 8-15.....	92,000	.....	.....	.....	.....	.....	.....
V-19-15.....	65,400	.....	.....	.....	.....	.....	.....

subsidence of the glandular enlargements during this period, and an almost complete disappearance of the posterior and anterior cervical glands followed the removal of adenoids by Dr. H. B. Hitz on June 26, 1913. Roentgen-ray treatments, benzol and sodium cacodylate have been given as seemed indicated. In general the patient's condition has remained fair during the two years that he has remained under observation. His principal complaint during this time has been lack of endurance and a feeling of weakness, but he has continued at his work whenever active treatments did not interfere. At times he has complained of pleuritic-like pains through the chest which either subsided without special treatment or seemed to disappear after X-ray treatment.

Blood smears from this case examined on March 5, 1915, when the leucocyte count was 60,000, showed 3% of polymorphonuclear neutrophils and 97% lymphocytes, of which 11% might be classified as large lymphocytes. On March 25 a group of glands was removed from the left axilla under the strictest aseptic precautions. The group was thoroughly flamed in a Bunsen burner and then incised and portions of the tissue were planted upon a variety of media, chiefly blood-serum and egg-medium. Some of the tubes thus planted were incubated aerobically, some anaerobically and still others under paraffin seal.

Of these inoculations six tubes remained sterile; four tubes showed a white coccus, one a white coccus and a diphtheroid organism, and one tube a pure culture of a diphtheroid organism, which grew apparently only upon the gland substance itself. By gradual transfers of gland substance, this diphtheroid was finally induced to grow upon Loeffler blood-serum, although at the time of writing it does not grow confluent, but as minute discrete colonies of a glistening whitish appearance. In stained smears it appears chiefly as a banded organism with marked tendency to the development of the clubbed forms. Coccoid forms are present, but the bacillary forms predominate. Morphologically it is identical with the strains isolated from cases of Hodgkin's disease. In its difficulty of cultivation upon artificial media, it resembles closely an organism grown by us from a case we designated "chloroma" because of greenish orbital tumors in addition to general glandular enlargement. Histologically the glands of the two cases are of almost identical appearance in their loss of architecture and diffuse infiltration with small lymphoid cells.

The second case studied presented clinically the appearance of a case of acute leukæmia, in the character of the mouth lesions, in the enlargement of certain cervical glands, and in the presence of cutaneous and subcutaneous tumors. However, blood counts during April and May have shown a leukopenia (5000-6000) with the following differential picture:

	Neut. %	Eos. %	Bas. %	Lymphocytes %	Trans. %
April 15, 1915, 6000.....	70.5	0.5	1.0	21.0	7.0
May 17, 1915, 5000.....	63.5	1.5	0.5	25.0	9.5
May 22, 1915, 5600.....	76.0	0.5	0.5	17.5	5.5

The history of the case is as follows:

April 14, 1915. A. K., æt. 52. Farmer.

*Complaint.*—Glandular enlargements in neck and groins.

*Family and Past History.*—Unimportant.

*Present Illness.*—Eight months ago after extraction of an ulcerated tooth he noted beginning enlargements in the glands beneath the jaw, which progressed and extended to the other side of the neck and to both groins. Later there appeared cutaneous and subcutaneous nodules, some becoming dark in color.

Four months ago the gums began to hypertrophy sufficiently to override the teeth.

He has continued to work, but has noted dyspnœa and palpitation after continued exertion.

*Physical Examination.*—A well nourished, muscular man, of good color. Skin soft and moist. There is a very extensive general lymphatic glandular enlargement with an involvement of the subcutaneous tissue leading to skin changes.

*Lymphatic Glands.*—Both triangles on both sides of the neck are quite completely filled with nodules varying in size from a cherry-pit to a walnut. In the lower parts these enlarged glands are discrete and movable; above they are matted together and fixed to the surrounding structures, particularly in the sub-maxillary region, where they seem to be almost subperiosteal. A few have become attached to the skin. Supra-orbital and pre-auricular enlargements are absent. Chains of glands can be felt extending downward on either side of the spine from the suboccipital region.

The left tonsil is distinctly hypertrophic. The submucous adenoid tissue, particularly on the left side, is so enlarged as to make the articulating surfaces of the teeth sink below the level of the gums. There is no distinct glandular enlargement at the base of the tongue. Marked pyorrhœa, caries and 'dribbling of saliva are present.

Both axillæ contain a few large glands and extending from these downward on to the sides of the chest and forward and upward in front of the pectoral muscles are chains of glands. These are attached deeply and the overlying skin is freely movable, giving an impression that the lymphatics leading into the thorax have been involved, though substernal distress is not manifest, nor are the superficial veins dilated.

Both brachial chains are enlarged above the epitrochlears. The inguinals are considerably enlarged but discrete and movable. Some glands above Poupart's ligament are palpable.

The liver and spleen are both impalpable and neither show enlargement on percussion. No ascites is detected.

The skin involvement is wide-spread from the forehead to the middle of the legs. These lesions seem to be of the progressive type, starting as subepithelial nodules, not tender or discolored. The epithelium wrinkles over these early nodules and when they are depressed assumes a pigskin appearance. Later reddening occurs and finally a dull purplish color develops. Now the epithelium has become adherent and looks like grain-leather. At times regression occurs in these nodules, leaving behind a macular-like discoloration. Ulceration has not occurred; no itching nor discomfort other than mechanical has been noted.

On April 15 Dr. R. H. Jackson excised a cutaneous nodule for culture and for histological examination. The cultures from this nodule resulted in the growth of a white coccus in three tubes and of a slow-growing diphtheroid and a rapid-growing coccus in a fourth. An attempt to isolate the diphtheroid failed.

On May 22 a second skin nodule was excised. The skin surface was seared in a Bunsen flame and the tissue dissected off from the under side of the nodule for culture.

This second attempt resulted in the growth of a diphtheroid in four tubes, a coccus in one, and no growth in five tubes. The organism is morphologically

like that found in the previous case, yet grows somewhat more freely upon artificial media. Its cultural characteristics have not yet been determined.

Histologically the skin nodule shows a diffuse infiltration, of the corium and subcutaneous tissue, by cells of the large mononuclear type, similar to those seen in the blood in acute leukæmia and those found in the lesions of lymphosarcoma. As in lymphosarcoma there appears to be no destruction of the infiltrated tissue. There is a slight fibroblastic stimulation.

The etiological relationship of this diphtheroid organism to the diseases described must perhaps remain for a time *sub judice*. The occurrence of similar organisms in a variety of diseases would certainly seem to be an argument against their etiological importance. However, it is our experience thus far that this type of organism is found with readiness only in one general group of apparently related diseases and in these without fail with good technique. In this group we would include Hodgkin's disease, the lymphogenous leukæmias, the pseudoleukæmias (lymphosarcoma), Banti's disease and probably also mycosis fungoides. Outside of this group, if the organism is found, there is also some histological evidence of its activity.

## THE EFFECT OF ROENTGEN RAYS ON THE RATE OF GROWTH OF SPONTANEOUS TUMORS IN MICE.

By JAMES B. MURPHY, M.D., AND JOHN J. MORTON, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, September 1, 1915.)

In a previous communication we reported the existence of a relationship between the resistant state to transplantable tumors in mice and a lymphocytic crisis in the circulating blood. We further demonstrated that by a previous destruction of the lymphoid tissue of these animals with x-ray a potentially resistant animal was rendered susceptible to cancer inoculation.<sup>1</sup>

In the course of some experiments on x-ray we have noted that the lymphoid elements, after extensive depletion by x-ray,<sup>2</sup> will soon start to regenerate actively. This process will continue, as has been noted before, to a period of overproduction of the lymphoid elements. The rapidity with which this occurs depends somewhat on the amount of original destruction and somewhat on the general condition of the animal. We have further noted that by one small dose of x-ray we could obtain in a certain proportion of animals a stimulation of the lymphoid elements, preceded by a comparatively short period in which the lymphocytes were below normal. This suggested an explanation of certain therapeutic effects of x-ray.

Our first problem was to determine whether or not x-rays in a small dose administered to an animal as a whole would produce an effect on the subsequent growth of a cancer, different from that produced by a similar dose applied directly to the cancer outside the body. For this purpose it was necessary in one set of animals to confine the x-ray effect to the animal alone, ruling out any possible action on the cancer, and, in a second set to confine the x-ray effect to the cancer, preventing an indirect effect on the animal. Spontaneous tumors of the mouse were selected for this work as a more

<sup>1</sup> Murphy, Jas. B., and Morton, J. J., *Jour. Exper. Med.*, 1915, xxii, 204.

<sup>2</sup> Heineke, H., *Mitt. a. d. Grenzgeb. d. Med. u. Chir.*, 1905, xiv, 21.

suitable material than the transplanted tumors, for reasons that will be explained later. The results are given here in a brief summary. A complete analysis of the size and characters of the cancers with autopsy findings will be given in a subsequent report.

*Series I.*—There were 52 mice with various stages and types of spontaneous cancers in this group. The tumors were removed as completely as possible by operation, and, with the cancer out, the whole animal was exposed to a stimulating dose of x-ray (Coolidge tube). Immediately afterwards a graft of the original cancer was replaced in the groin of the animal. In 26 of the 52 animals treated in this fashion, there resulted a complete immunity to the recurrence of the disease. Only those animals were included in this number that lived and remained in good physical condition for at least five weeks after the treatment. The majority lived from two to four months, some to eight months, and some are still living. There has been no evidence of a local recurrence at the site of operation, nor where the graft was implanted, or of metastasis in those that have died. Among the remaining 26 animals of the series the average time for the appearance of the graft was five weeks and four days, a figure which contrasts strongly with the figure for the control animals. The number of recurrences at the original location of the tumor was 11 among the 52 animals, all occurring in the latter 26.

*Series II.*—For a control series we had 29 mice with spontaneous tumors of various sorts. These were operated on in the same manner as the animals in the first series, but a graft of the cancer was returned without treatment to either the animal or the cancer. The tumors were kept outside the body for the same length of time as in the first series. In 28 of the 29 the grafts grew progressively. In one the graft grew for a period and then retrogressed to complete absorption. The average time for the grafts to become palpable was one week and five days. This is about the same figure obtained by Rous.<sup>3</sup> Local recurrences of the cancer occurred in 14 of the 29 animals.

*Series III.*—The cancers in these animals were removed in the same manner as in the first two series, but in this group the cancers were subjected to the same amount of treatment that the animals had

<sup>3</sup> Rous, P., *Jour. Exper. Med.*, 1914, xx, 433.



received in the first series. A graft from the cancer, after this treatment outside of the body, was returned to the groin of the original host, as in the other experiments. 10 mice with spontaneous tumors were used for this series, and in all 10 the returned grafts grew. The average time for these to become palpable was one week and three days. There was a local recurrence of the tumor in 4 of the 10 animals.

A tabulation of the figures for the three experiments is given for comparison in Table I.

TABLE I.

	Immune.	Susceptible.	Local recurrence of tumor.	Average time for ap- pearance of graft.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
Series I*.....	50.0	50.0	21.2	5 wks., 4 days.
" II.....	3.4	96.6	48.3	1 wk., 5 "
" III.....	0.0	100	40.0	1 " 3 "

\* Series I, animals treated by x-ray, while cancer was out. Later a graft of the tumor was returned. Series II, control animals in which cancer was removed and a graft returned without treatment to either animal or tumor. Series III, cancers removed and subjected direct to x-ray treatment and a graft returned to the original host.

It will be seen from the figures in Table I that x-ray administered directly to the cancer outside of the body is insufficient to prevent the growth of a graft returned to the original host. On the other hand, the same small dose of x-ray given to the animal with the cancer removed was sufficient to render 50 per cent of the mice so treated immune to returned untreated grafts of their own tumors and greatly retard the growth in the other 50 per cent.

The contrast between the control animals with 1 immune out of 29, and the x-rayed series with 26 immune out of 52 is striking, as is also the comparison of local recurrence in the two series. The delayed appearance of the graft in the treated series is important and this period could perhaps have been prolonged or a recurrence prevented altogether by a second exposure to x-ray after a suitable interval.

Total white counts and differentials were done on all of these mice

before operation<sup>4</sup> or treatment and on part of them at intervals after. So far in the limited number counted systematically afterward, our treatment has given in the successful cases a definite increase in the lymphocytes. Whether or not this increase is vitally concerned in the immunity process to spontaneous tumors is a point to which at present we are unwilling to commit ourselves. The results in the light of our previous experiments are strongly suggestive of this, however.

We have demonstrated a direct effect of x-rays on the animal, which renders it more highly resistant to replants of its own cancer than would normally be the case. There is also an absence of any demonstrable effect of this small dose of x-ray when administered to the cancer direct. Grafts of such tumors when returned to their original host grow as well as do the controls.<sup>5</sup>

<sup>4</sup> Counts made on over 100 untreated mice with spontaneous tumors have failed to show an abnormally low lymphocytic content in the circulating blood. This result is contrary to that obtained by Baeslack (Baeslack, F. W., *Ztschr. f. Immunitätsforsch., Orig.*, 1913-14, xx, 421) in counts on two mice with spontaneous tumors. We were also unable to confirm his reported decline in the numbers of lymphocytes in animals with growing transplantable cancers (Murphy and Morton, *loc. cit.*). He also gives differential counts on four mice with natural immunity to transplanted tumors, but as no total white cell counts are given it is impossible to tell whether his percentage variations are due to fluctuations in the polymorphonuclear cell or in the lymphocyte.

<sup>5</sup> We have avoided in this communication any discussion of the massive and contradictory literature on direct x-ray effects. We are unaware of any experiments that bring out the above points.

## PRODUCTION OF PNEUMONIC LESIONS BY INTRA- BRONCHIAL INSUFFLATION OF UNORGAN- IZED SUBSTANCES.

By B. S. KLINE AND S. J. MELTZER.

*(From the Department of Physiology and Pharmacology of The Rockefeller  
Institute for Medical Research.)*

Earlier communications from this department have shown that intrabronchial insufflations of virulent pneumococci in dogs produce lobar pneumonia similar in every respect to the lesions of lobar pneumonia as observed in human beings. In later experiments, Wollstein and Meltzer demonstrated that, at least macroscopically, typical pneumonic lesions can be produced also by avirulent pneumococci and by the saprophytic bacillus megatherium. Microscopically it was established that the lesions produced by the virulent pneumococci contained a great amount of fibrin, while the lesions produced by the last-named organisms (avirulent pneumococci and bacillus megatherium) contained only very little fibrin.

In the present series of experiments various unorganized substances were insufflated into the bronchi of dogs which were killed after twenty-four or forty-eight hours. The substances were: aleuronat suspension in starch solution (autoclaved), starch solution, egg yolk, lecithin, egg white and cholesterin. The results were striking and are as follows: aleuronat, starch, egg yolk and lecithin produced lesions which macroscopically could not be distinguished from those produced by insufflation of pneumococci. Egg white never produced pneumonic lesions of any extent and even in the occasional patches many of the bronchioles and alveoli containing this protein showed microscopically no nucleated cells whatever in their lumina. The few patches produced by cholesterin were associated with the bronchioles and adjoining alveoli. We shall not describe here the nature of the histological pictures of the lesions

under discussion; we shall merely mention the fact that those lesions produced by aleuronat and starch were similar microscopically to those produced by virulent pneumococci, that is, the lesion contained in addition to an exudate of similar cells, etc., a good deal of fibrin; while lesions produced by egg yolk and lecithin resembled more those produced by avirulent pneumococci and bacillus megatherium.

In brief, we may say that our experiments have demonstrated definitely that the hepatization of the lungs, similar to the one observed macroscopically in lobar pneumonia, can be produced by such unorganized substances as aleuronat, starch, egg yolk and lecithin, while egg white does not produce such an effect. The following chart illustrates our results:

Substance Injected Intrabronchially.	No. of Animals.	Lesion.		Lung Culture.
		Patches of Consolidation.	Lobar Type of Consolidation.	
Aleuronat in starch solution . . . . .	8		+	8 sterile.
Do. . . . .	2	+		2 "
Starch solution . . . . .	7		+	6 "
Egg yolk . . . . .	11		+	7 " (1?).
Do. . . . .	1	+		"
Lecithin . . . . .	6		+	4 "
Egg white . . . . .	5	-		5 "
Do. . . . .	4	+		4 "

## ON *d*-LYXOHEXOSAMINIC ACID AND ON $\alpha$ - $\alpha_1$ -ANHYDRO-MUCIC ACID.

BY P. A. LEVENE AND F. B. LA FORGE.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, July 9, 1915.)

Theoretically there are possible as many  $\alpha$ -amino hexoses as there are hexoses. In order to simplify the present argument the enantiomorphous isomerism of the entire molecule will not be taken into consideration. The hexoses will be discussed as if they belonged to one series, either *d* or *l*. Under such conditions the number of hexoses and of  $\alpha$ -hexosamines is eight. For each hexosamine there is possible a corresponding  $\alpha$ -hexosaminic acid, while the number of the possible  $\alpha$ - $\alpha_1$ -anhydro-tetrahydroxyadipic acids is only six.

Of the eight possible  $\alpha$ -hexosamines two are known. Of these one (glucosamine) was obtained from natural sources<sup>1</sup> and synthetically,<sup>2</sup> and the other (chondrosamine) was discovered recently by the present writers.<sup>3</sup> Of the eight possible hexosaminic acids four were known prior to the present work: glucosaminic acid (obtained from glucosamine and synthetically from arabinose),<sup>4</sup> chondrosaminic acid (obtained by us from chondrosamine), xylohexosaminic, and ribohexosaminic acids (obtained by us synthetically).<sup>5</sup>

There is a smaller gap in the series of possible  $\alpha$ - $\alpha_1$ -anhydro-tetrahydroxyadipic acids, since, prior to this publication, only one was missing of the six possible. The five known acids were: the so called "isosaccharic acid," which was proven by the present writ-

<sup>1</sup> Ledderhose, G., *Ztschr. f. physiol. Chem.*, 1878-79, ii, 213; 1880, iv, 139.

<sup>2</sup> Fischer, E., and Leuchs, H., *Ber. d. deutsch. chem. Gesellsch.*, 1903, xxxvi, 24.

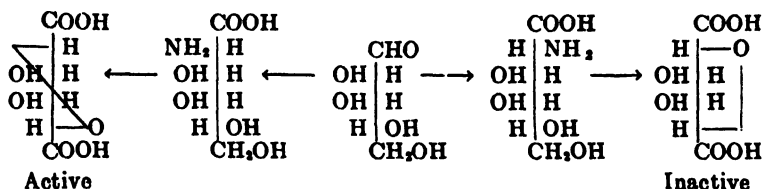
<sup>3</sup> Levene, P. A., and La Forge, F. B., *Jour. Biol. Chem.*, 1914, xviii, 123.

<sup>4</sup> Fischer, E., and Tiemann, F., *Ber. d. deutsch. chem. Gesellsch.*, 1894, xxvii, 138. Fischer and Leuchs, *ibid.*, 1902, xxxv, 3787.

<sup>5</sup> Levene and La Forge, *Jour. Biol. Chem.*, 1915, xx, 433; 1915, xxi, 351.

ers to be  $\alpha$ - $\alpha_1$ -anhydro-mannosaccharic; further,  $\alpha$ - $\alpha_1$ -anhydro-saccharic,  $\alpha$ - $\alpha_1$ -anhydro-idosaccharic,  $\alpha$ - $\alpha_1$ -anhydro-allomucic,  $\alpha$ - $\alpha_1$ -anhydro-talomucic acids.<sup>5</sup>

The present work deals with the preparation of one more hexosaminic acid, namely, *d*-lyxohexosaminic acid, and of  $\alpha$ - $\alpha_1$ -anhydro-mucic acid. The amino-acid was obtained by the action of hydrocyanic acid on lyxosimine. The anhydro-tetrahydroxyadipic acid was obtained by deamination of the amino-acid and subsequent oxidation with nitric acid. The structure of the acid was established by the fact that it was found to be optically inactive. Of the two epimeric anhydro-tetrahydroxyadipic acids derived from *d*-lyxohexosaminic acid, one only, namely, the one corresponding to mucic, is optically inactive; the other is identical with anhydro-talomucic acid and has been obtained by us from chondrosamine.



Thus the series of anhydro-tetrahydroxyadipic acids is completed.

Referring to the preparation of the  $\alpha$ - $\alpha_1$ -anhydro-mucic acid it must be emphasized that the amino-acid needs to be absolutely pure in order that it may yield a satisfactory quantity of the anhydro-mucic acid.

Reference may also be made to the mode of preparation of lyxose. Starting out from milk sugar it was found convenient to isolate first the galactose by an unpublished process originated in the Bureau of Chemistry, Washington, D. C. The galactonic acid was isolated directly as the calcium salt, which in turn was readily converted into the pentose by Ruff's method.<sup>6</sup>

#### EXPERIMENTAL.

##### *Preparation of Acid Calcium d-Galactonate.*

1600 grams *d*-galactose were dissolved in four parts of water,

<sup>6</sup> Ruff, O., and Ollendorff, G., *Ber. d. deutsch. chem. Gesellsch.*, 1900, xxxiii. 1798.

2000 grams bromine added and allowed to stand three days, with occasional shaking at room temperature. The solution was then concentrated in vacuum to about two-thirds of its volume, to remove the excess of bromine. The hydrobromic acid was removed with lead carbonate, the filtrate freed from lead with hydrogen sulphide and concentrated to about 3 liters in vacuum. It was then boiled with an excess of calcium carbonate for about three-quarters of an hour and filtered hot on a Buchner funnel. On cooling, the calcium salt crystallized out in an almost chemically pure condition. For the preparation of lyxose it was recrystallized once from hot water. Final yield: 1500 grams.

*d-Lyxosimine.*

100 grams crystalline finely ground *d*-lyxose were dissolved in 100 cc. dry methyl alcoholic ammonia saturated at about  $+10^{\circ}$ . Crystallization of the imine began in a few minutes and was complete after about forty-eight hours. The product was chemically pure and needed only to be washed with a little methyl alcoholic ammonia and dried. The yield was quantitative; melting point,  $142-143^{\circ}$  (uncorrected).

0.1932 gm. of substance gave 12.6 cc.  $\frac{N}{N}$   $\text{NH}_3$ .

	Calculated for $\text{C}_5\text{H}_{11}\text{NO}_4$ :	Found:
N .....	9.40	9.12

*d-Lyxohexosaminic Acid.*

32 grams of finely divided lyxosimine were covered with 32 cc. of water, 18 cc. of 80 per cent hydrocyanic acid added, and the suspension was warmed carefully to about  $35^{\circ}$  by immersion in warm water. Solution took place rapidly and soon spontaneous warming of the reaction mixture began. The temperature was controlled by a thermometer placed in the reaction flask and prevented from rising above  $42^{\circ}$ . When the tendency to warm up had ceased, the solution was allowed to stand for about one-half hour at room temperature, cooled to  $0^{\circ}$ , mixed with 300 cc. cold concentrated hydrochloric acid, and saturated with hydrochloric acid gas.

Three portions, as above, were combined and worked up exactly

as in the case of xylohexosaminic acid.<sup>7</sup> Care must be taken in handling the final concentrated aqueous solution. If too great an excess of methyl alcohol is added, it carries down gummy impurities, which are hard to remove from the crystals. Hence the alcohol should be added very carefully. From 95 grams imine the yield was 18 grams. It was recrystallized from 50 per cent methyl alcohol. *d*-Lyxohexosaminic acid darkens slowly above 180° and decomposes at about 215°.

0.1217 gm. of substance gave 15.3 cc. amino N, at 21.5°, 762 mm.

0.1036 gm. of substance gave 0.1382 gm. CO<sub>2</sub> and 0.0632 gm. H<sub>2</sub>O.

	Calculated for C <sub>6</sub> H <sub>11</sub> O <sub>6</sub> N:	Found:
N .....	7.18	7.12
C .....	36.92	36.54
H .....	6.66	6.85

The rotation of the substance in 2.5 per cent hydrochloric acid solution was as follows:

$$[\alpha]_D^{25}, \text{ after 15 minutes} = \frac{-0.25^\circ}{1} \times \frac{2.1662}{0.1505} = -3.58^\circ$$

$$[\alpha]_D^{25}, \text{ after 44 hours} = \frac{-1.44^\circ}{1} \times \frac{2.1662}{0.1505} = -20.72^\circ$$

#### *α-α<sub>1</sub>-Anhydro-Mucic Acid.*

9 grams of lyxohexosaminic acid were dissolved in 100 cc. 3 per cent hydrochloric acid and 15 grams of silver nitrite added in portions over a period of about four hours. A few cc. of 10 per cent hydrochloric acid were then added and the reaction was allowed to proceed over night at room temperature. The silver chloride was filtered off and all but a slight excess of the hydrochloric acid removed from the filtrate with silver nitrate. The clear solution was concentrated *in vacuo* to about 25 cc., 30 cc. of concentrated nitric acid were added, and the solution was warmed in an Erlenmeyer flask over a small flame until a vigorous evolution of red fumes began. The flame was then removed and the reaction allowed to proceed with occasional warming. After twelve minutes the solution was poured into two glass dishes and evaporated to a thick syrup on the water bath. The contents of the dishes were then diluted with a little water, combined, and again concentrated. After

<sup>7</sup> Levene and La Forge, *Jour. Biol. Chem.*, 1915, xxi, 354.



evaporating once more with water, the syrup on cooling solidified to a mass of white crystals which appeared under the microscope as white oblong plates. After standing in the refrigerator for a time the mass was triturated with a few cc. of a mixture of dry acetone and ether and filtered on a Buchner funnel. The process was repeated once more to remove the adhering syrup. The crude product after drying weighed about 2 grams, although more crystallized from the acetone-ether mixture used for drying. For analysis it was recrystallized by dissolving in about one hundred parts of boiling dry acetone, filtering and concentrating the solution to about one-eighth of its volume. Anhydro-mucic acid crystallizes from acetone without crystal water in long prisms which melt at  $203-204^{\circ}$  (corrected).<sup>8</sup>

0.1040 gm. of substance required 10.7 cc.  $\frac{N}{10}$  KOH; calculated, 10.8 cc.

0.1563 gm. of substance in 3 cc.  $H_2O$  showed no rotation with  $D$ -light in a 1 dm. tube, where a rotation of  $0.02^{\circ}$  would not have escaped detection.

0.1424 gm. of substance gave 0.1946 gm.  $CO_2$  and 0.0534 gm.  $H_2O$ .

	Calculated for $C_6H_8O_7$ :	Found:
C .....	37.50	37.27
H .....	4.20	4.17

<sup>8</sup> Temperature given for anhydro-allomucic,  $200-201^{\circ}$ , also corrected.

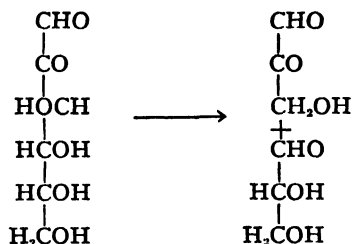
## ON THE ACTION OF ASEPTIC TISSUE ON GLUCOSONE.

By P. A. LEVENE AND G. M. MEYER.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, July 9, 1915.)

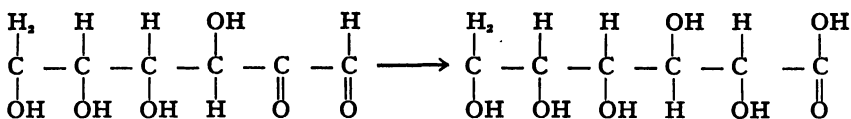
It is definitely established by the work of recent years that in the process of animal oxidation the hexose molecule suffers transformation into lactic acid, and that this phase is preceded by the transformation of one molecule of hexose into two of methyl glyoxal. In a previous communication the possible steps leading to this transformation were discussed.<sup>1</sup> It was shown in that publication that whenever any one hydrogen atom of the hexose molecule was substituted by a complex organic or mineral radicle the sugar was not affected by the tissue enzymes. In the present experiments it was aimed to test the action of the tissue enzymes on glucosone. Theoretically glucosone may dissociate into one molecule of glyceric aldehyde and one of hydroxymethyl glyoxal, and these in turn may be transformed into lactic (through methyl glyoxal) and pyruvic acids respectively.



On the other hand, glucosone may be regarded as a substituted glyoxal, and by analogy with methyl glyoxal might have been expected to be transformed into a hexonic acid:<sup>2</sup>

<sup>1</sup> Levene, P. A., and Meyer, G. M., *Jour. Biol. Chem.*, 1914, xviii, 469.

<sup>2</sup> Dakin, H. D., and Dudley, H. W., *Jour. Biol. Chem.*, 1913, xiv, 155. Levene and Meyer, *ibid.*, 1913, xiv, 151 and 551. Neuberg, C., *Biochem. Ztschr.*, 1913, xlix, 502.



The experiments were performed under the same conditions as in the previous work. Aseptic kidney tissue was used as enzyme. In all experiments the glucosone solution remained unchanged. The reducing power for Fehling's and for permanganate solutions was not altered by the action of kidney tissue. Also the yield of phenyl osazone was apparently not affected by the experiment.

Thus the results of the present experiments are in harmony with those of last year. They show that only the unchanged hexose molecule is affected by the tissue enzyme, and that only the unchanged hexose undergoes a cleavage into two molecules, each containing a chain of three carbons. This in its turn may signify that the first step in the process of lactic acid formation from sugar consists in the dissociation of the hexose molecule into two of glyceric aldehyde. Perhaps this phase is the part of a special enzyme.

#### EXPERIMENTAL.

**Tissues.**—Kidneys were removed under aseptic conditions from exsanguinated rabbits.

**Solutions.**—For the preparation of glucosone 60 gm. of phenyl glucosazone in portions of 15 gm. were decomposed with 150 cc. concentrated HCl and the glucosone was isolated according to the method of Fischer.<sup>3</sup> The glucosone was obtained as a clear syrup. With phenyl hydrazine it formed an osazone in the cold. Approximately 3 gm. of this syrup were dissolved in 150 cc. sterile 1 per cent Henderson phosphate solution and passed through a sterile Berkefeld filter. This solution was divided into three portions of 50 cc. each. One rabbit kidney was cut into small pieces and added to each flask. Two of the flasks were allowed to stand at 37° for three hours; the other served as control.

**Bacteriological Control.**—Smears and cultures were made of all tissue mixtures before analysis and only those free from contamination were considered. The bacterial examinations were made by Dr. Martha Wollstein, to whom we desire to express our appreciation.

**Methods of Analysis.**—The diluted solutions were freed of proteins by small amounts of metaphosphoric acid. The presence of unchanged glucosone was noted in all solutions after standing at 37°.

**Oxidation with Fehling's Solution.**—The Lehmann-Maquenne method as described by Griesbach and Strassner<sup>4</sup> was employed.

<sup>3</sup> Fischer, E., *Ber. d. deutsch. chem. Gesellsch.*, 1889, xxii, 87.

<sup>4</sup> Griesbach, W., and Strassner, H., *Ztschr. f. physiol. Chem.*, 1913, lxxxviii, 199.

*Oxidation with Permanganate.*—The total oxidation as described by Greifenhagen, König, and Scholl, and outlined in a previous communication, was also used.<sup>5</sup>

*Oxidation with Fehling's Solution.*

	Original solution.	CuSO <sub>4</sub> .	$\frac{N}{10}$ Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> .	CuSO <sub>4</sub> used.	Equivalent glucose.	Glucose.
	cc.	cc.	cc.	cc.	mg.	per cent
I Control .....	2	27.8	21.8	6.0	19.0	0.95
After 18 hrs. ....	2	27.8	21.8	6.0	19.0	0.95
II Control .....	4	27.8	17.5	10.3	33.2	0.83
After 18 hrs. ....	4	27.8	17.4	10.4	33.6	0.84
After 42 hrs. ....	4	27.8	17.3	10.4	33.6	0.84
III Control .....	4	27.8	17.3	10.5	34.0	0.85
After 18 hrs. ....	4	27.8	17.2	10.6	34.3	0.86
After 42 hrs. ....	4	27.8	17.3	10.5	34.0	0.85

*Oxidation with Permanganate.*

	Original solution.	$\frac{N}{10}$ KMnO <sub>4</sub>	$\frac{N}{10}$ oxalic acid.	$\frac{N}{10}$ KMnO <sub>4</sub> used.	Glucosone.	Glucose.
	cc.	cc.	cc.	cc.	mg.	per cent
I Control .....	2	41.00	22.6	18.4	30.1	1.51
After 18 hrs. ....	2	41.30	22.7	18.6	30.4	1.52
II Control.....	2	40.00	23.8	16.2	26.5	1.33
After 18 hrs. ....	2	41.25	24.5	16.6	27.1	1.35
After 42 hrs. ....	2	40.65	23.3	16.3	26.6	1.33
III Control.....	2	40.90	24.1	16.8	27.5	1.38
After 18 hrs. ....	2	41.00	23.9	17.1	27.9	1.39
After 42 hrs. ....	2	43.00	26.1	16.9	27.6	1.38

\* 1 cc.  $\frac{N}{10}$  KMnO<sub>4</sub> = 1.634 mg. glucosone.

<sup>5</sup> Levene and Meyer, *Jour. Biol. Chem.*, 1912, xii, 268.

## ON THE KYRINE FRACTION OBTAINED ON PARTIAL HYDROLYSIS OF PROTEINS.

### SECOND COMMUNICATION.

BY P. A. LEVENE AND J. VAN DER SCHEER.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, July 14, 1915.)

The significance of the theoretical assumptions based on Siegfried's original conception of the composition of kyrines has been discussed in an earlier paper.<sup>1</sup> According to Siegfried the kyrines are fragments of the protein molecule—polypeptides, resembling in every respect the natural protamines, and as such carry evidence in favor of the assumption that the molecule of every protein is built around a protamine nucleus. Levene and Birchard succeeded in fractionating the kyrine fraction of gelatin into two peptides, one containing in its molecule only arginine and the other only lysine. One of the two was obtained in a state of satisfactory purity. The work was since then extended to the study of the kyrine fraction of casein. The object of the work was twofold. First, it was realized that the kyrines obtained from other sources than gelatin might actually represent the large fragments of the structure assumed by Siegfried. On the other hand, it was possible that most kyrines were composed of simple basic peptides. In that case, a comparative study of their structure could be expected to furnish some information as to the distinctive structure of the molecule of individual proteins.

The procedure employed for fractionating the gelatin kyrine was applied in a general way also for fractionation of the substance obtained from casein. Only one additional step was introduced. In the earlier work the kyrine was separated into one part form-

<sup>1</sup> Levene, P. A., and Birchard, F. J., *Jour. Biol. Chem.*, 1912-13, xiii, 277.

ing a silver complex insoluble in water, while the other part was soluble in hot water, the other a phosphotungstate which was insol-fractionated into two parts, the one forming a phosphotungstate soluble in hot water, the other a phosphotungstate which was insol-uble. The details of the procedure remained as in the previous work.

From one of the fractions it was possible to obtain a sulphate which had all the macroscopic appearance and character of a crystal-line substance. It was no longer hygroscopic as were Siegfried's kyrines, or the amorphous peptides. It was composed of long, glis-tening needles. Under the microscope they presented in part per-fect prismatic needles, in part tapering formations resembling Leh-mann's liquid crystals (Fig. 1). By means of fractional crystalliza-tion this substance could not be altered in its elementary composition nor in its ratio of  $\frac{\text{amino N}}{\text{total N}}$ .

The ratio prior to hydrolysis was 50 per cent, and after hydrolysis, 75 per cent.

Furthermore, it was found that all the basic nitrogen was pres-ent in the form of amino nitrogen, hence the peptide contained in its molecule only one basic substance: lysine. This assumption was substantiated by the fact that the lysine could be isolated in the form of its picrate in a quantity required by the theoretical assump-tion. Hence there remains no doubt that the substance was a tripep-tide containing lysine, and two amino-acids, of which one did not contain a primary  $\text{NH}_2$  group and therefore might have been either proline or oxyproline. The elementary analysis of the sulphate had a complete agreement with the assumption that the tripeptide was composed of lysine, oxyproline, and valine. The theory for this assumption requires  $\text{C} = 42.20$ ,  $\text{H} = 7.04$ ,  $\text{S} = 7.03$ , and the figures found by analysis were  $\text{C} = 42.29$ ,  $\text{H} = 6.97$ ,  $\text{S} = 6.60$ .

Unfortunately we were not equally successful in the isolation and in the identification of the amino-acids as we were in that of lysine. Valine was obtained once in a rather small quantity; oxyproline has not been identified up to the present. This perhaps is due to the fact that in its pure form the substance was available for hydrolysis in every experiment in a rather small quantity. Furthermore, the

possibility is not excluded that the sulphate consisted of two, or of several, lysine peptides.

However, the principal fact was established, namely that the casein kyrine can also be fractionated into simple peptides each containing in its molecule only one basic substance.



FIG. 1.

#### EXPERIMENTAL.

500 grams of casein were hydrolyzed with 5.5 liters of 12.5 per cent hydrochloric acid for five weeks at 40°C. At the end of that time the solution was filtered, diluted with water to twice its volume, and to the solution phosphotungstic acid was added as long as a precipitate formed. After standing in the refrigerator over night, the phosphotungstic precipitate was filtered off and washed repeatedly with cold 5 per cent sulphuric acid until the wash water was free

from hydrochloric acid. The precipitate was then dissolved in 5 liters of 10 per cent ammonia, the solution brought to a boil, and the phosphotungstic acid removed by means of finely powdered barium hydrate. The excess of barium hydrate was removed with carbon dioxide and the filtrate concentrated to a small volume.

This material was further fractionated by means of silver nitrate and barium hydrate.

The solution was first slightly acidified with dilute nitric acid and then silver nitrate was added in slight excess. To the solution barium hydrate was then added until no more precipitate was formed. Both filtrate and precipitate were acidified with sulphuric acid and decomposed with hydrogen sulphide, and, after filtering, neutralized with barium hydrate and concentrated. The peptides were precipitated with phosphotungstic acid and the precipitates freed from nitric acid by repeatedly washing with cold 5 per cent sulphuric acid.

The phosphotungstic acid precipitates were then extracted four times with large volumes of boiling hot water. The phosphotungstic acid was removed from the aqueous solution by lead acetate and the excess of lead by hydrogen sulphide. The phosphotungstic precipitates were dissolved in ammonia and freed from phosphotungstic acid in the way previously described. Thus four fractions were obtained, namely:

Silver precipitate .....	{ 1. Phosphotungstate soluble in hot water.
	2. Phosphotungstate extracted with hot water.
Filtrate from silver nitrate	{ 3. Phosphotungstate soluble in hot water.
treatment .....	4. Phosphotungstate extracted with hot water.

Fraction 4, obtained after the treatment with silver nitrate and barium hydrate and extraction of its phosphotungstic precipitate with hot water, had the ratio of amino nitrogen to total nitrogen of about 1 to 3, which ratio did not change after repeatedly fractionating it by means of silver nitrate and barium hydrate.

The final solution was concentrated to a small volume, acidified with sulphuric acid, and precipitated in about 6 liters of 99.8 per cent alcohol. The precipitate of the sulphate was filtered off, redissolved in a small amount of water, and again precipitated in alcohol. It was found that on repeating the process the ratio of amino nitrogen to total nitrogen had increased after each precipi-



tation until it reached the value of 0.50, after which two precipitations in alcohol did not give any marked change in the nitrogen ratio.

### *Amino Nitrogen Ratio in the Purified Sulphate.*

The ratio of amino nitrogen to total nitrogen was determined according to the method of Van Slyke.

0.6000 gm. of sulphate was dissolved in 20 cc. of water.

Total N: 5 cc. of solution neutralized 10.6 cc.  $\frac{N}{10}$   $H_2SO_4$ . N = 14.84 mg.

Amino N: 5 cc. of solution gave 13.20 cc. gas at 23°, 768 mm. N = 7.47 mg.

Amino N	.....	50.5 per cent.
Total N	.....	

### *Analysis of the Substance.*

I. 0.1098 gm. of substance gave 0.1664 gm.  $CO_2$  and 0.0668 gm.  $H_2O$ .

0.0668 gm. of substance gave 0.0310 gm.  $BaSO_4$ .

II. 0.0929 gm. of substance gave 0.1450 gm.  $CO_2$  and 0.0604 gm.  $H_2O$ .

	Calculated for $C_{15}H_{22}N_4SO_7$ :	Found:	
		I.	II.
C .....	42.20	42.29	42.58
H .....	7.04	6.97	7.33
S .....	7.03	6.60	

### *Hydrolysis: Nitrogen Distribution.*

2 grams of the sulphate were hydrolyzed for twenty-four hours with 50 cc. of 20 per cent hydrochloric acid, the excess of hydrochloric acid was then distilled off *in vacuo*, and the solution made up to 100 cc.

Total N: 5 cc. of solution neutralized 6.95 cc.  $\frac{N}{10}$   $H_2SO_4$ . N = 9.73 mg.

Amino N: 5 cc. of solution gave 13.10 cc. gas at 22°, 763 mm. N = 7.40 mg.

Amino N	.....	76 per cent.
Total N	.....	

From this it is evident that after hydrolysis among the components there was present a substance containing nitrogen in a different form from a primary amino group. To that class belong proline, oxyproline, tryptophane, arginine, and histidine. Further hydrolysis has shown that all the basic nitrogen was composed of amino nitrogen, and hence was made up of lysine.

*Hydrolysis Aiming to Show the Quantity of Lysine in the Peptide.*

Another 20 grams of hydrolyzed sulphate were, after removal of the hydrochloric acid and sulphuric acid as before, made up to 500 cc., and 75 cc. of concentrated sulphuric acid and 1 liter of 10 per cent phosphotungstic acid solution added. After standing in the refrigerator the phosphotungstic precipitate was filtered off and the phosphotungstic acid removed in the usual way by means of barium hydrate.

Of the 100 cc. solution 2 cc. were taken, the ammonia was boiled off with calcium oxide, and the solution made up to 10 cc.

Total N: 5 cc. solution neutralized 3.10 cc.  $\frac{N}{14}$   $H_2SO_4$ . N = 4.34 mg.

Amino N: 2 cc. solution gave 3.10 cc. gas at 25°, 765 mm. N = 1.73 mg.

Amino N

Total N ..... 99.9 per cent.

Total 100 cc. contain 0.4320 gm. of amino N, corresponding to 2.27 gm. of lysine.

The solution was concentrated to 50 cc. and 3 grams of picric acid were added. On boiling, a precipitate was formed which was filtered off, washed with ether, and dried. Yield: 5.6 grams, corresponding to 2.3 grams of lysine.

0.1500 gm. of the picrate gave 20.20 cc. N gas in the Van Slyke apparatus, at 22°, 765 mm.

	Calculated for $C_6H_{14}N_2O_2 \cdot C_6H_5(NO_2)_3OH$ :	Found:
N .....	7.80	7.65

Hence it is made clear that the substance contained only one base, and that this was lysine.

*Analysis of the Mono-Amino-Acids.*

10 grams of the sulphate were hydrolyzed by boiling for twenty-four hours with 200 cc. of 20 per cent hydrochloric acid; the sulphuric acid was removed with barium hydrate and the hydrochloric acid distilled off under diminished pressure. The remaining hydrochloric acid was removed by means of silver sulphate. The silver was removed finally with hydrogen sulphide and the sulphuric acid quantitatively with barium hydrate.

It was attempted to separate the amino-acids by converting them

into the copper salts. This process was not successful. Hence the lysine was removed by means of phosphotungstic acid. The filtrate was freed from the excess phosphotungstic acid by means of barium hydrate. This again was removed quantitatively by means of sulphuric acid. The solution was concentrated to a very small volume. Absolute alcohol was then added to the solution. On standing, a precipitate formed which was filtered over a small Buchner funnel, washed with alcohol, and recrystallized from a minimal quantity of water. The yield was 0.200 gram.

Amino N : 0.020 gm. of substance gave 4.00 cc. gas at 21°, 768 mm. N = 2.28 mg.  
The substance contained 6.37 per cent ash.

0.0970 gm. of substance gave 0.1841 gm. CO<sub>2</sub> and 0.0792 gm. H<sub>2</sub>O.

	Calculated for C <sub>6</sub> H <sub>11</sub> NO <sub>2</sub> :	Found:
C .....	51.30	51.68
H .....	9.40	9.11
Amino N .....	11.95	12.17

The substance had the following rotation:

$$[\alpha]_D^{20} = \frac{+0.70^\circ \times 2.8885}{0.5 \times 1.055 \times 0.1500} = +25.54^\circ$$

#### *Attempt to Isolate $\alpha$ -Proline or Oxyproline.*

The product of hydrolysis of 10 grams of the sulphate was precipitated with phosphotungstic acid and the filtrate freed from phosphotungstic acid with barium hydrate in the usual way. In the barium-free solution the amino-acids were converted into their copper salts. From the mixture  $\alpha$ -proline could not be isolated. Hence the product was freed from copper, the solution was made up to a volume of 80 cc. to which 3 grams of barium hydrate were added, and heated in a sealed tube at 140–150° for five hours. The attempt to isolate the copper salt of the inactive  $\alpha$ -proline was not successful.

In another experiment with 10 grams of the sulphate an attempt was made to isolate oxyproline. The product was treated with phosphotungstic acid, and the amino-acid fraction was converted into the copper salts. The process which had been successfully employed in this laboratory on a previous occasion was followed in this experiment.<sup>2</sup> The copper salts were fractionated by means of dilute alcohol (80 per cent) but no pure oxyproline could be isolated.

<sup>2</sup> Levene, P. A., and Beatty, W. A., *Ztschr. f. physiol. Chem.*, 1906, xlix, 256.

## SYNTHESIS OF NORMAL TRIDECYCLIC AND TETRACOSANIC ACIDS.

BY P. A. LEVENE, C. J. WEST, C. H. ALLEN, AND J. VAN DER SCHEER.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, September 27, 1915.)

In a previous communication<sup>1</sup> Levene and West reported the results of a renewed investigation into the melting points of several of the higher normal fatty acids. The change in the melting points of the acids with the increase of the number of carbon atoms in the chain was found consistent with the generally accepted rule. Only tridecylic acid showed a much higher melting point than would be expected. It was mentioned in that article that it was intended to make this discrepancy the subject of a separate investigation.

In the previous work tridecylic acid was prepared by oxidation of  $\alpha$ -hydroxy-myristic acid. It is evident that the acid prepared by this method may contain traces of myristic acid, if there was exercised a lack of care in the purification of the  $\alpha$ -bromo- or  $\alpha$ -hydroxy-myristic acids. It was, therefore, concluded to synthesize the acid by the malonic ester method.

The acid obtained in this manner had a melting point of 44.5-45.5° (corrected). In order to secure greater certainty regarding this finding on the tridecylic acid obtained through the malonic ester synthesis the substance was prepared independently by two of the present authors. The physical properties of the two substances were identical.

It was then concluded to repeat the preparation of the tridecylic acid by the oxidation of  $\alpha$ -hydroxy-myristic acid. In this instance care was taken to remove completely traces of myristic acid. The acid prepared from the pure hydroxy-myristic acid had the same melting point as that obtained through malonic ester.

<sup>1</sup> Levene, P. A., and West, C. J., *Jour. Biol. Chem.*, 1914, xviii, 463.

The tetracosanic acid was prepared because of the bearing the acid had on the structure of lignoceric acid. Meyer, Brod, and Soyka<sup>2</sup> were the first to express the view that lignoceric acid differed in structure from the normal tetracosanic acid. They prepared the latter synthetically and determined its melting point. According to them the melting point exceeded that of behenic acid only by 1.5–2°. Since the authors failed to isolate the intermediate product, there always remained some doubt as to the absolute purity of their product.

In this work the melting points were determined in a sulphuric ionic acid served for the preparation of tetracosanic acid. Recrystallized from toluene the substance had the melting point of 87.5–88°. The acid prepared by Meyer, Brod, and Soyka melted at 85.5–86°.

In this work the melting points were determined in a sulphuric acid bath provided with a stirrer; the rate of heating was uniform—7 to 8 seconds for each degree.

#### EXPERIMENTAL PART.

##### *Tridecylic Acid.*

*Undecylic Alcohol*,<sup>3</sup>  $C_{11}H_{23}OH$ .—Undecylic alcohol was prepared by reducing 50 grams of ethyl undecylate with 35 grams of absolute ethyl alcohol and 32 grams of metallic sodium. The yield was 23 grams of alcohol, which boiled at 147° at 25 mm. pressure.

*Undecylic Iodide*,  $C_{11}H_{23}I$ .—60 grams of undecylic alcohol, 45 grams of iodine, and 8 grams of red phosphorus were heated one and one-half hours at 180°. The reaction product was taken up in ether, the solution shaken with aqueous sodium thiosulphate, and dried. 85 grams of iodide were obtained, boiling at 125° at 3 mm. pressure, and at 117° at 1.3 mm. pressure.

0.1663 gm. of iodide gave 0.1378 gm. AgI (Carius).

	Calculated for $C_{11}H_{23}I$ :	Found:
I .....	44.99	44.79

<sup>2</sup> Meyer, H., Brod, L., and Soyka, W., *Monatsh. f. Chem.*, 1913, xxxiv, 1133.

<sup>3</sup> Jeffreys, E., *Am. Chem. Jour.*, 1899, xxii, 37.

*Ethyl Undecylmalonate*,  $C_{11}H_{23}CH(COOC_2H_5)_2$ .—The condensation was carried out as follows: 2.05 grams of sodium were dissolved in 50 cc. of absolute ethyl alcohol, 14.2 grams of ethyl malonate and 25 grams of undecylic iodide were added, and the mixture was heated three hours on the water bath under a reflux. The reaction product was treated with water, the ester extracted with ether, the ether solution washed with water and dried. Ethyl undecylmalonate boils at 208–209° (corrected) at 21 mm. pressure.

0.3506 mg. of ester required 21.0 cc.  $\frac{N}{10}$  NaOH for saponification. Calculated, 22.4 cc.

*Undecylmalonic Acid*,  $C_{11}H_{23}CH(CO_2H)_2$ .—For the preparation of the dibasic acid it is unnecessary to distill the ester. The crude product, obtained upon concentrating the ether solution, was saponified by warming with an excess of 50 per cent sodium hydroxide. The soap was washed twice with dry acetone and then decomposed with concentrated hydrochloric acid. The free acid was extracted, taken up in acetone, the solution filtered, the acetone removed on the steam bath, the product washed with petroleum ether and crystallized from benzene and then from a mixture of acetone and petroleum ether. It melts at 108.5° (corrected) without decomposition.

0.300 gm. of substance, dissolved in a mixture of ethyl alcohol and benzene, required 22.78 cc.  $\frac{N}{10}$  NaOH for neutralization.

	Calculated for $C_{11}H_{23}O_4$	Found:
Mol. Wt. ....	258.2	263.4

*Tridecylic Acid*,  $C_{12}H_{25}COOH$ .—The dibasic acid was heated at 180° for one hour or until the evolution of carbon dioxide had practically ceased. The resulting tridecylic acid was then distilled over in vacuum. Two fractions were collected, the first boiling between 148° and 153° at 1.2 mm. pressure, the second, between 153° and 158° at the same pressure. A second experiment gave a product boiling at 199–200° at 24 mm. pressure (corrected). Each fraction was twice crystallized out of dry acetone. The tridecylic acid thus obtained melted at 44.5–45.5° (corrected).

0.500 gm. of substance, as above, neutralized 23.4 cc.  $\frac{N}{10}$  NaOH.

	Calculated for $C_{13}H_{26}O_2$ :	Found:
Mol. Wt. ....	214.2	213.7

In order to determine whether the method or the material was at fault in our earlier work, we repeated the preparation of tridecylic acid by the oxidation of  $\alpha$ -hydroxy-myristic acid with potassium permanganate. The acid thus obtained was identical with the synthetic product from undecylic acid.

1.00 gm. of acid, as above, neutralized 47 cc.  $\frac{N}{10}$  NaOH.

	Calculated:	Found:
Mol. Wt. ....	214.2	212.8

This indicates that the method is satisfactory, and shows that with pure hydroxy acids pure monobasic acids may be obtained.

*Tetracosanic Acid*,  $C_{23}H_{47}COOH$ .

*Ethyl Docosylmalonate*,  $C_{22}H_{45}CH(COOC_2H_5)_2$ .—0.53 gram of sodium was dissolved in 50 cc. of absolute ethyl alcohol and to the cooled solution 3.68 grams of ethyl malonate and 10 grams of docosyl iodide,  $C_{22}H_{45}I$ , were added. The mixture was heated on the water bath for twenty-four hours. The reaction product was diluted with water. The ester which separated out was washed with water and recrystallized twice from acetone. The yield was about 10 grams. The analyzed product was obtained by esterifying a sample of docosylmalonic acid which had been twice crystallized out of acetone. It melts at  $48^\circ$  (corrected).

0.1026 gm. of substance gave 0.2798 gm.  $CO_2$  and 0.1138 gm.  $H_2O$ .

	Calculated for $C_{23}H_{47}O_4$ :	Found:
C .....	74.29	74.38
H .....	12.08	12.45

*Docosylmalonic Acid*,  $C_{22}H_{45}CH(CO_2H)_2$ .—The above ester was dissolved in boiling alcohol and about five equivalents of 50 per cent sodium hydroxide were added, and the mixture was boiled. The soap which separated out upon cooling the alcohol was filtered off, washed with water, and then extracted thoroughly with boiling

acetone. The free acid was liberated by treating the soap with concentrated hydrochloric acid. This was purified by recrystallizing it from acetone. Various methods were tried to obtain a product which would give a correct molecular weight. As this was not readily accomplished, due to the difficulty of removing the last traces of alkali, the dibasic acid was changed into the monobasic after two recrystallizations from acetone.

*Ethyl Tetracosanate*,  $C_{23}H_{47}CO_2C_2H_5$ .—Docosylmalonic acid was heated one hour at  $160-180^\circ$  and the crude reaction product esterified by boiling with 5 per cent sulphuric acid. After repeating the esterification three times the ester was recrystallized twice out of acetone, distilled in vacuum, again recrystallized, distilled, and recrystallized. The product then boiled at  $118^\circ$  at 0.6 mm. pressure and melted at  $56-57^\circ$  (corrected).

0.100 gm. of substance gave 0.2893 gm.  $CO_2$  and 0.1173 gm.  $H_2O$ .

	Calculated for $C_{25}H_{50}O_2$ :	Found:
C .....	78.79	78.90
H .....	13.13	13.15

*Tetracosanic Acid*,  $C_{24}H_{48}O_2$ ,  $C_{23}H_{47}COOH$ .—The ethyl ester was dissolved in boiling alcohol, and five times the equivalent of sodium hydroxide added for saponification. The soap was washed with water and thoroughly extracted with boiling acetone. The acid, which was liberated by boiling with concentrated hydrochloric acid, was purified through the lead salt. From toluene, tetracosanic acid crystallizes in scales, melting at  $87.5-88^\circ$ . Meyer gives  $85.5-86^\circ$ .

0.400 gm. of acid neutralized 10.9 cc.  $\frac{N}{10}$  NaOH.

	Calculated for $C_{24}H_{48}O_2$ :	Found:
Mol. Wt. ....	368.5	367



## THE RELATION BETWEEN THE CONFIGURATION AND ROTATION OF EPIMERIC MONO-CARBOXYLIC SUGAR ACIDS.

By P. A. LEVENE.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, September 27, 1915.)

Hudson<sup>1</sup> was the first to discover certain relationships between the configuration and the rotatory power of sugars, glucosides, and the lactones of sugar acids. By so doing he also has brought telling evidence in support of the principle of optical superposition, at least for the sugars and sugar derivatives. Accepting the correctness of the principle one may apply it to the differentiation of the mono-carboxylic acids of two epimeric sugars in the following way.

Two epimeric acids differ only by the configuration of their  $\alpha$ -carbon atoms. These are antipodes. Accepting the magnitude of the optical rotation of this carbon atom as equal to A and that of the sum of the other asymmetric carbon atoms as equal to B, the rotation of one acid is  $+A + B = M$  and that of the epimeric form is  $-A + B = N$  (M and N being the empirical values). Hence,

$$B = \frac{M + N}{2}, \quad +A = \frac{2M - M - N}{2} \quad \text{or} \quad \frac{M - N}{2},$$

and

$$-A = \frac{2N - M - N}{2} \quad \text{or} \quad -\left(\frac{M - N}{2}\right).$$

However, the free acids as a rule are unstable in aqueous solution, passing into their lactones, and therefore the observations will have to be made on their salts. The present literature presents only few data regarding the optical activity of salts of epimeric acids, but in

<sup>1</sup>Hudson, C. S., *Jour. Am. Chem. Soc.*, 1909, xxxi, 66; 1910, xxxii, 338; 1911, xxxiii, 405. Anderson, E., *ibid.*, 1911, xxxiii, 1510; 1912, xxxiv, 51.

every instance when the salts of the same base of two epimeric acids have been obtained it was found that for those acids in which the hydroxyl of the  $\alpha$ -carbon atom had the same position as in *d*-gluconic acid, the calculated value for the rotation of the  $\alpha$ -carbon atom had the sign *plus*; and for those having the configuration of the hydroxyl of the  $\alpha$ -carbon atom corresponding to *d*-mannonic acid the sign was *minus*. Hence this method may be employed to determine the configuration of sugar acids when chemical methods are for some reason or other not applicable. It is hoped that the principle will be found of service for determining the structure of the  $\alpha$ -hexosamines.

The following data are taken from the work of Nef.<sup>2</sup>

Acid.	Formula.	Derivative.	$[\alpha]_D$ .	Calculated rotation of the $\alpha$ -carbon atom.
<i>d</i> -Gluconic...	$\text{CH}_2\text{OH} \begin{array}{c} \text{H} \text{ H OH} \text{ H} \\ \text{OH OH} \text{ H OH} \end{array} \text{COOH}$	Calcium salt	+10.5	+9.0
<i>d</i> -Mannonic .	$\text{CH}_2\text{OH} \begin{array}{c} \text{H} \text{ H OH} \text{ OH} \\ \text{OH OH} \text{ H H} \end{array} \text{COOH}$	Calcium salt	-7.5	-9.0
<i>d</i> -Gluconic...	$\text{CH}_2\text{OH} \begin{array}{c} \text{H} \text{ H OH} \text{ H} \\ \text{OH OH} \text{ H OH} \end{array} \text{COOH}$	Strychnine salt	-18.76	+3.97
<i>d</i> -Mannonic .	$\text{CH}_2\text{OH} \begin{array}{c} \text{H} \text{ H OH} \text{ OH} \\ \text{OH OH} \text{ H H} \end{array} \text{COOH}$	Strychnine salt	-26.7	-3.97
<i>d</i> -Gulonic*...	$\text{CH}_2\text{OH} \begin{array}{c} \text{H OH} \text{ H H} \\ \text{OH H OH OH} \end{array} \text{COOH}$	Phenylhydrazide	+13.4	+12.91
<i>d</i> -Idonic*...	$\text{CH}_2\text{OH} \begin{array}{c} \text{H OH} \text{ H OH} \\ \text{OH H OH H} \end{array} \text{COOH}$	Phenylhydrazide	-12.42	-12.91
<i>d</i> -Gulonic...	$\text{CH}_2\text{OH} \begin{array}{c} \text{H OH} \text{ H H} \\ \text{OH H OH OH} \end{array} \text{COOH}$	Strychnine salt	-17.24	+4.38
<i>d</i> -Idonic....	$\text{CH}_2\text{OH} \begin{array}{c} \text{H OH} \text{ H OH} \\ \text{OH H OH H} \end{array} \text{COOH}$	Strychnine salt	-26.00	-4.38
<i>d</i> -Xylonic* .	$\text{CH}_2\text{OH} \begin{array}{c} \text{H OH} \text{ H} \\ \text{OH H OH} \end{array} \text{COOH}$	Brucine salt	-18.70	+4.43
<i>d</i> -Lyxonic...	$\text{CH}_2\text{OH} \begin{array}{c} \text{H OH} \text{ OH} \\ \text{OH H H} \end{array} \text{COOH}$	Brucine salt	-27.57	-4.43

\* Rosanoff's nomenclature.

Dr. G. M. Meyer and the writer are engaged in extending the study to a larger number of salts of the sugar acids.

<sup>2</sup> Nef, J. U., *Ann. d. Chem.*, 1914, cccci, 204.

## A CONTRIBUTION TO THE BIOLOGY OF PERIPHERAL NERVES IN TRANSPLANTATION.<sup>1</sup>

By RÅGNVALD INGEBRIGTSEN, M.D.

(*From the Pathological Institute of the University Clinic, Christiania.*)

PLATES 44 TO 46.

(Received for publication, May 11, 1915.)

The large amount of experimental and clinical work in transplantation that has been done during the last few years has elucidated many essential points, and the transplantation of bone, skin, connective tissue, blood vessels, and glandular organs has assumed a practical value. This is not the case with peripheral nerves, possibly because their transplantation in human beings has been mainly heteroplastic.

The processes leading to the degeneration of a divided peripheral nerve are well known, and the work of Nageotte has recently added minute and important histological details to our knowledge of the cells of Schwann and the part played by them in the degeneration and regeneration of a peripheral nerve. I shall not comment upon attempts at transplantation of nerves in human beings. Of the experimental work in this field I shall mention only the results of those investigators who have made histological examinations of the transplanted pieces, especially for the Wallerian degeneration, and who have attempted, in the case of a negative result, to determine the variations in these processes; that is, in the Wallerian degeneration, produced by transplantation.

Huber<sup>2</sup> has examined histologically mainly heteroplastic transplanted segments of nerves, and concluded from his experiments that the degenerative process, which occurred in these pieces was very much like the true Wallerian degeneration, the various stages of which, however, succeeded each other more rapidly in the graft than in the peripheral part of a divided nerve.

<sup>1</sup> Aided by a grant from The Rockefeller Institute for Medical Research.

<sup>2</sup> Huber, G. C., *Jour. Morphol.*, 1895, xi, 629.

Ballance and Stewart<sup>3</sup> in their experiments on transplantation are reserved in their conclusions, expressing themselves as follows: The degeneration appears in the graft exactly as in the peripheral part of a divided nerve. The graft itself is a piece of dead tissue and is gradually absorbed and replaced like a clot by living tissue. The regeneration occurs later, but is not a result of the work of the cells of the graft itself. This conclusion seems to me to express two conflicting views, of which one, if true, necessarily excludes the other. If the degeneration of the graft appears exactly as in the peripheral segment of a divided nerve, then the segment is not dead. For the peripheral segment of a divided nerve is not dead. The axis cylinder dies and degenerates, but the cells of Schwann, on the other hand, after the division give evidence of their life by their proliferation.

Neither Ballance and Stewart nor Huber discriminated between the auto-, homo-, and heteroplastic transplantation. This was first done by Merzbacher.<sup>4</sup> In 1905 Merzbacher observed that the graft in auto- and homoplastic transplantations only degenerated in a typical way, whereas in heteroplastic transplantations there occurred in the fibers various regressive processes that resulted in the necrosis of the piece. In auto- and homotransplantations the grafts survive and therefore are capable of a true Wallerian degeneration, which can take place only in nerves in a condition of survival. In heterotransplantations the grafts die and cannot degenerate, but become necrotic; in these cases there is no, or only an insignificant formation of myelin ovoids. The conclusions of Merzbacher were confirmed by the experiments of Segale.<sup>5</sup>

Verga<sup>6</sup> in 1910 performed a series of homo- and heterotransplantations, bridging the central and peripheral part of a divided nerve by the graft. Verga found that the segments healed and always degenerated, and that from an anatomical standpoint there was no difference between the homo- and the heteroplastic graft.<sup>7</sup>

In 1911 Maccabruni<sup>8</sup> in Golgi's laboratory made a number of homo- and heteroplastic transplantations. His results are in accord with those of Huber and Verga, recording a typical Wallerian degeneration in homoplastic as well as in heteroplastic grafts. The center of both kinds of graft he found necrotic, possibly due to the lacking supply of nourishment. Thin grafts degenerated completely without necrosis. From the 8th to the 14th day he found proliferation of the cells inside the nerve fibers by means of karyokinetic division. The process has a slower course than in a degenerating nerve. Regarding the origin of the cellular elements inside the fibers, Maccabruni expresses some reservation. His preparations do not show whether these cells represent the syncytium of Schwann, or whether they are connective tissue cells.

<sup>3</sup> Ballance, C. A., and Stewart, P., *Rev. neurol.*, 1902, x, 860.

<sup>4</sup> Merzbacher, *Neurol. Centralbl.*, 1905, xxiv, 150.

<sup>5</sup> Segale, L., cited by Maccabruni, F., *Folia neuro-biol.*, 1911, v, 598.

<sup>6</sup> Verga, *Jahresber. f. Chir.*, 1910, xvi, 481.

<sup>7</sup> This publication is not available. In the summary of the article in the *Jahresber. f. Chir.* the cells of Schwann are not mentioned.

<sup>8</sup> Maccabruni, *loc. cit.*

Besides the work just mentioned, experimental transplantation of nerves has been performed by Gluck,<sup>9</sup> Kilvington,<sup>10</sup> and Duroux.<sup>11</sup> The results of these investigators are encouraging as far as the function is concerned. Neither Kilvington nor Duroux, however, made histological examinations of the transplanted pieces, and the interpretation and the conclusions drawn from his material by Gluck concerning the processes of regeneration are not convincing. The lack of proof, in the work of Gluck, that the graft is different from dead material has caused Kölliker to remark that the nerve bridge must be supposed to prevent the regeneration of the peripheral part instead of facilitating it, and Kölliker advocates the bridging of the defect by strands of catgut or tubes as superior to the application of a graft of nervous tissue.

In the problem of transplantation of nerves the question of the fate and survival and multiplication of the cells of Schwann is of importance. The solution of this point, which is the only reliable sign of the survival of the transplanted piece, gives the key to the problem and will influence the procedure of surgeons in cases of nerve defects. If the grafts die and become necrotic they are no more suitable for bridges than strands of catgut. If it is true, on the other hand, that the grafts do survive, the statement of Kölliker lacks support, and in bridging nerve defects grafts of peripheral nerves must be preferred to dead material.

I have experimented on the sciatic nerve of rabbits, from which pieces 2 to 3 cm. long are taken out and then either reimplanted into the same animal, united to the cut ends of the nerve by means of a single silk suture (autoplastic), or implanted into the sciatic nerve of another rabbit (homoplastic), or into guinea pigs (heteroplastic). I have made three series of experiments. In each series I have operated on several animals and the transplanted pieces were removed for histological examination at different intervals (4, 8, 12, 16, or 20 days) after the transplantation.

Then the pieces were treated in the way indicated by Nageotte, which has given excellent results in his study of the Wallerian degeneration. The grafts were hardened in Dominici's solution, next they were dissociated by means of needles as far as possible, and stained by hematoxylin before passing through alcohol and mounted in cedar oil. From some cases sections were prepared and treated according to the method of Marchi.

<sup>9</sup> Gluck, *Jahresber. f. Chir.*, 1895, i, 282.

<sup>10</sup> Kilvington, B., *Brit. Med. Jour.*, 1908, i, 1414.

<sup>11</sup> Duroux, E., *Lyon Chir.*, 1912, viii, 562.

I wish to emphasize the necessity of making dissociation preparations, if one wishes to make indisputable observations on the cells of Schwann. In such preparations only we are sure that a certain cell belongs to a certain fiber, and in this case only can we count the accurate number of cells in each individual fiber. I shall give a summary of my results from each of the three series, beginning with the autoplasmic transplantations.

### *Autoplasmic Transplantations.*

In this series the examination of the graft four and six days after transplantation (Fig. 4) reveals a process which is not very different from the ordinary Wallerian degeneration (Figs. 1, 2, and 3). The nuclei of the cells of Schwann have fallen in towards the center of the fibers between two myelin ovoids. The nuclei are richly provided with chromatin and are embedded in protoplasm. In a few fibers from the sixth day two or three nuclei are observed close to each other, indicating that multiplication of these cells has already begun. Between and in the myelin ovoids there are immigrated mononuclear cells of a type quite different from the cells of Schwann. The nuclei of these cells are smaller and richer in chromatin than the nuclei of the cells of Schwann. They present phagocytic properties filling their cell bodies with fragments of myelin, and Nageotte, who found them in the Wallerian degeneration, called them "*corps granuleux*." Probably they are lymphocytes.

The only difference between the autoplasmic graft from the fourth and sixth days and the peripheral part of a divided nerve is that in the latter the formation of myelin ovoids is more advanced than in the graft. In the examination of the grafts in later stages, we find this feature again and again. After eight days we find a degenerative process resembling a somewhat delayed Wallerian degeneration. A graft from the eighth day is in as degenerative a stage as a peripheral nerve from the fifth to sixth days. But in some fibers we observe no degeneration at all; there is no formation of myelin ovoids and they look perfectly normal. These fibers belong to the central parts of the graft, and judging from the appearance

of Marchi preparations, these fibers later become necrotic. This is true about the central fibers of homoplastic grafts as well as autoplasmic. On the twelfth day we find as pronounced a Wallerian degeneration as on the eighth or ninth day. A large number of myelin ovoids has been formed, and there are many "*corps granuleux*" and numerous nuclei of the cells of Schwann.

After the sixteenth and twentieth days (Figs. 5 and 6) the nuclei of the cells of Schwann are arranged in long rows inside the sheaths of Schwann with continuous protoplasmic bridges about and between them; the only difference from the Wallerian degeneration of the same stage is that in the latter the absorption of myelin fragments is a good deal more advanced than in the graft.

### *Homoplastic Transplantations.*

I shall next describe the results of my homoplastic transplantations.

In almost every respect the preparations from the fourth and fifth days (Figs. 7 and 8) resemble the picture of a nerve on the fourth and fifth days of Wallerian degeneration. The nuclei of the cells of Schwann have fallen in towards the center of the fibers, and are richly provided with chromatin. A formation of myelin ovoids has started and only a few immigrated cells are seen. In one of the preparations from the fourth day we find a cell of particular interest. In one of the fibers (Fig. 8) we observe three oval nuclei of Schwann close to each other, and also a large darkly stained cell including a nucleus in mitotic division. This cell gives proof that the cells of Schwann multiply in a homoplastic transplanted graft. The increased number and the long rows of nuclei of Schwann in individual fibers already give strong evidence for the probability of such a conclusion, but the mitotic figure presents a picture from a stage of the process itself. We do not hesitate in the identification of this cell. The well outlined protoplasmic body of the cell, which is never observed in the immigrated cells during their mitotic division, determines that the cell is really a cell of Schwann.

I have never observed in the grafts in autoplasmic transplantation such a mitotic division of the cells of Schwann. But mitotic divi-

sions are not easily found and are not frequently seen in the cells of Schwann during their proliferation in the ordinary Wallerian degeneration. Accordingly there is no reason to doubt that mitotic divisions may also be observed in autoplasmic transplantations, since we know that grafts in such conditions are best fit for survival.

During the first ten to eleven days the degenerative process in the homoplasmic transplanted grafts appears mainly as in autoplasmic transplantations; that is, like a Wallerian degeneration, only a little more slowly. Myelin ovoids are formed, the cells of Schwann multiply, and immigrated phagocytic cells loaded with fatty granules are seen in the fibers (Fig. 9). From the eleventh to twelfth days, these cells are present in a number considerably exceeding those in the autoplasmic grafts. They are steadily increasing in number, and from the sixteenth to eighteenth days (Fig. 10) they form a marked feature in the whole picture.

It is possible that the presence of these numerous immigrated cells in homoplasmic grafts—cells provided with phagocytic properties and probably of lymphocytic origin—are playing some part in the mechanism of immunity against homoplasmic transplantation of tissue in general. For in transplantation of organs and tissue there is a marked difference between the final result of homoplasmic and autoplasmic transplantation (the kidneys, for instance). But I do not wish to enter upon further discussion of the importance of the phagocytic cells. I wish only to add that in homoplasmic nerve grafts the nuclei of Schwann from the eighteenth to twentieth days are pale and faintly stained and are evidently in a necrobiotic condition, which is possibly dependent upon the presence of the lymphocytes.

#### *Heteroplasmic Transplantations.*

In heteroplasmic transplanted nerves an abundant formation of myelin ovoids occurs during the first four to five days. But later these grafts do not resemble either the autoplasmic or homoplasmic transplanted pieces or nerves in Wallerian degeneration. There is no proliferation of the cells of Schwann. These cells, on the contrary, are faintly stained or have completely disappeared. From the eighth and tenth days the contents of the fibers consist mostly of



irregularly broken up pieces and fragments of myelin and protoplasm and the whole fiber looks necrotic (Fig. 11).

In later stages we find between and in the fibers numerous immigrated cells, and from the sixteenth to eighteenth days the graft on gross examination is yellowish, soft, and necrotic, or it is encapsulated by young connective tissue.

#### SUMMARY.

In autoplasmic transplanted nerves a degenerative process occurs which resembles the ordinary Wallerian degeneration, but appears a little more slowly than the latter. The cells of Schwann are in a condition of survival and are capable of multiplication after the transplantation.

In homoplastic transplanted nerves I have found a degenerative process resembling a Wallerian degeneration, somewhat delayed. The cells of Schwann multiply, and for some time at least are in a condition of survival. After twelve to fourteen days an abundant and increasing immigration of lymphocytes is observed, and from the eighteenth day the cells of Schwann develop a necrobiotic appearance.

In heteroplastic transplanted nerves numerous myelin ovoids are formed during the first four to five days, but there is no proliferation of the cells of Schwann, and no Wallerian degeneration is seen. The graft becomes necrotic within about two weeks.

The formation of ovoids that occurs during the first four to five days after the performance of the heteroplastic transplantation does not reveal the condition of the life of the graft. This formation of myelin ovoids is found in the nerve fibers when they have been kept in an incubator for twenty-four hours in Ringer solution (Nageotte<sup>12</sup>) or in homologous or heterologous serum, but it is not found in the fibers after their incubation in isotonic salt solution (Nageotte), the presence of calcium being necessary for the occurrence of ovoid formation.

<sup>12</sup> Nageotte, J., *Compt. rend. Soc. de biol.*, 1910, lxix, 556.

## CONCLUSIONS.

Heteroplastic transplanted nerves become necrotic. They are unsuitable for bridges in cases of nerve defects, and my results explain the failure of the attempts at heteroplastic transplantation of nerves in human beings.

If we wish to bridge a nerve defect by implantation we must use autoplasmic or homoplastic grafts. The occurrence of a Wallerian degeneration in these grafts during the first two to three weeks after the transplantation should make bridging a promising operation; for in this period the grafts resemble the peripheral part of a divided nerve and must be assumed to be capable of regeneration, and thus are very different from dead material.

I have studied the process of regeneration, and shall communicate in a future article my results of bridging defects, which are encouraging as far as the function is concerned.

My results with homoplastic transplantation of nerves have a bearing on the homoplastic transplantation of limbs, which has been successfully performed in dogs by Carrel. None of his dogs lived long enough to show any function of the transplanted leg. The practical value of this operation is dependent, of course, upon the return of function, and especially on the regeneration of the nerves in the transplanted leg. The results with homoplastic transplantation of nerves seem to indicate the possibility of a regeneration of the nerves in a homoplastic transplanted leg.

## EXPLANATION OF PLATES.

## PLATE 44.

Figs. 1, 2, and 3 are nerve fibers from the peripheral part of divided nerves. Wallerian degeneration in different stages.

FIG. 1. Wallerian degeneration, 4th day.

FIG. 2. Wallerian degeneration, 7th day.

FIG. 3. Wallerian degeneration, 14th day. Multiplication of the nuclei of Schwann, numerous immigrated cells ("*corps granuleux*").

FIG. 4. Nerve fibers from a graft, 6 days after transplantation (autoplasmic). Multiplication of the nuclei of Schwann.

FIG. 5. Nerve fiber from a graft, 16 days after transplantation (autoplasmic). Long rows of nuclei of Schwann. Some immigrated cells ("*corps granuleux*").

## PLATE 45.

FIG. 6. Nerve fiber from a graft, 18 days after transplantation (autoplastic). Multiplication of the nuclei of Schwann. Reduction of the myelin ovoids. Numerous immigrated cells.

FIG. 7. Nerve fibers from a graft, 4 days after transplantation (homoplastic). The nuclei of Schwann have fallen in towards the center of the fibers, embedded in protoplasm.

FIG. 8. Nerve fibers from a graft, 4 days after transplantation (homoplastic). In one of the fibers a mitotic figure is seen in a cell of Schwann.

FIG. 9. Nerve fibers from a graft, 10 days after transplantation (homoplastic). Multiplication of the nuclei of Schwann. Immigrated small darkly stained cells ("*corps granuleux*").

## PLATE 46.

FIG. 10. Nerve fiber from a graft, 18 days after transplantation (homoplastic). Multiplication of the nuclei of Schwann. These nuclei are pale and faintly stained. A large number of immigrated cells is seen.

FIG. 11. Nerve fibers from a graft, 10 days after transplantation (heteroplastic). No cell is seen. The fibers appear necrotic.

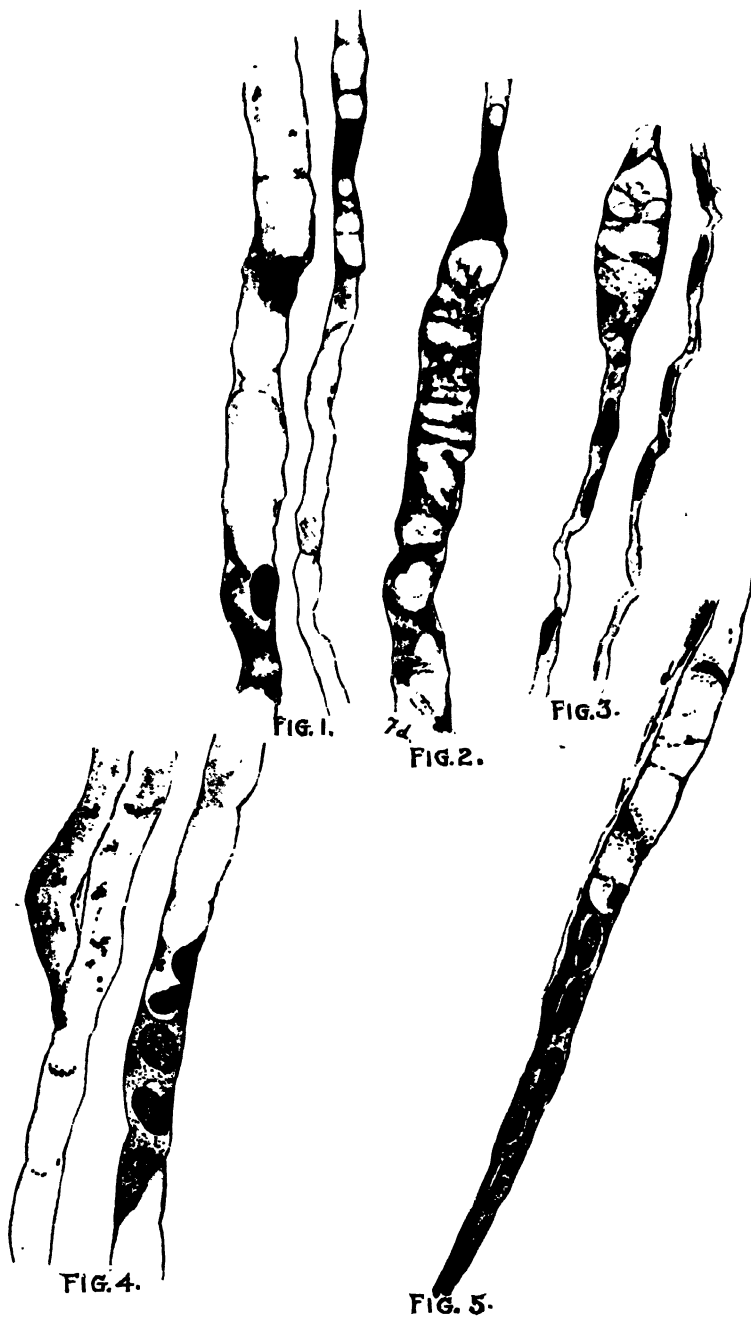






FIG. 6.



FIG. 7.

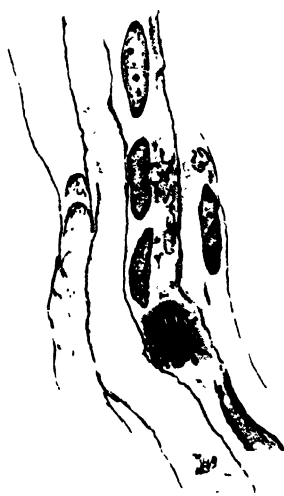


FIG. 8.



FIG. 9.

(Ingebrigtsen: Biology of Peripheral Nerves in Transplantation.)



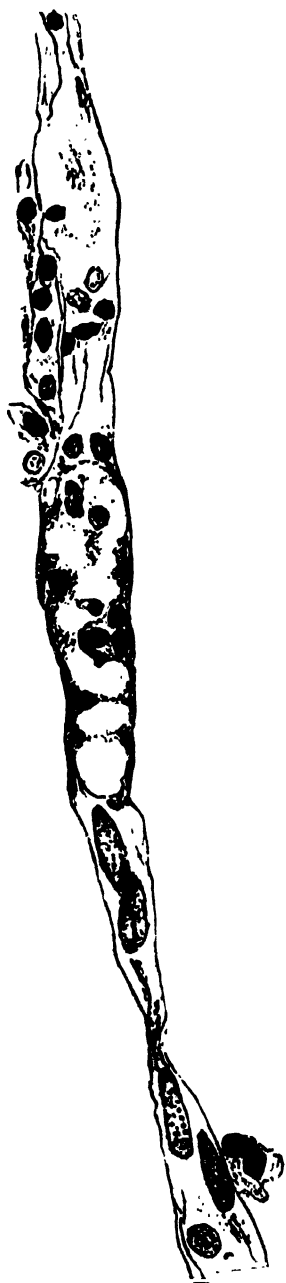


FIG. 10.



FIG. 11.

(Ingebrigtsen: Biology of Peripheral Nerves in Transplantation.)





## RULES AND MECHANISM OF INHIBITION AND CORRELATION IN THE REGENERATION OF BRYOPHYLLUM CALYGINUM.

By JACQUES LOEB.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

### I. INTRODUCTION.

In the phenomena of regeneration the problem of correlation appears, that is, the influence of the whole on the part. A part cut out from a whole organism may regenerate, while no such regeneration will occur so long as the part is not separated from the whole. What are the forces inherent in the whole which exercise the control over the part resulting in the prevention of regeneration?

We cannot form a definite idea of this inhibitory mechanism until we know the laws or rules underlying this prevention of regeneration or growth in the normal plant. Only if we succeed in finding such rules and if they are sufficiently simple can we with any hope of success begin to draw conclusions concerning the nature of the mechanism underlying these phenomena of inhibition and correlation. The reason why it is difficult to find such laws lies in the fact that the phenomena of regeneration in most organisms are too complicated or too indeterminate for such a purpose, and we are compelled to look for an organism which is especially favorable for such a purpose. The tropical plant *Bryophyllum calycinum* is apparently such an organism, and the writer has succeeded in finding some rules governing the phenomena of inhibition and correlation of growth. These rules are so simple and transparent that they form, in the opinion of the writer, a securer basis for hypothesis than is offered by most former experiments in this direction which have not led to such simple rules.

The advantage of this plant for the study of the problem of

regeneration lies in the fact that shoots can grow out only from definitely located buds in the stem and in the notches of the leaf. The "Anlagen" of roots are not so definitely located, and roots may grow out apparently from practically any spot on the stem of the plant; they are, therefore, not so appropriate for the establishment of definite and simple rules of inhibition, and their growth will not be considered in this paper.

One bud is located in each notch of a leaf of *Bryophyllum calycinum*; when such a notch begins to grow it forms first roots and later shoots. It is well known that if the leaves of this plant are cut off and put on moist soil (or suspended in moist air) they will form roots and shoots from their notches. This is the mode of propagation of this plant. The question is: Why does a leaf not form roots and shoots in its notches so long as it is in connection with a healthy plant? The buds in the notches of the leaf are not the only ones which are inhibited from growing when forming parts of the whole; the buds on the stem, one of which is found in the axilla of each of the two leaves in each node, are in the same condition, and the same question may be raised, namely: Why do not these buds grow out as long as they form part of a plant, while if isolated they may grow into shoots?

A very few words will suffice to show that the stimulus of the wound is not responsible for the growing out of buds, though the conditions at the edge of the wound are responsible for the healing or covering of the wound by the spreading of epithelial cells over the area laid free by the wound; and they may possibly be directly responsible for the callus formation in the case of plants. When we break off a leaf of *Bryophyllum*, the notches of the leaf will grow out into roots and shoots, but these notches are far away from the cut end of the stalk of the leaf. Moreover, as a rule, the notches in the middle of the leaf will grow out first, and not those nearest the wound caused by the cutting or breaking off of the leaf. It is plainly impossible, therefore, to connect in any way the growth of the notches of a leaf with the "stimulus" of the wound. The same may be said for the growth of roots in the main stem of the plant, which may take place several inches away from the seat of injury. We need not dwell on this point any further, since this is generally

conceded. It is chiefly in animals that we find regeneration localized at the wound; but this is apparently due to the fact that in such animals any cells may give rise to new growth, while in *Bryophyllum calycinum* the power of giving rise to shoots is restricted to buds located in definite places in the plant.

## II. Isolation as the Cause of Regeneration.

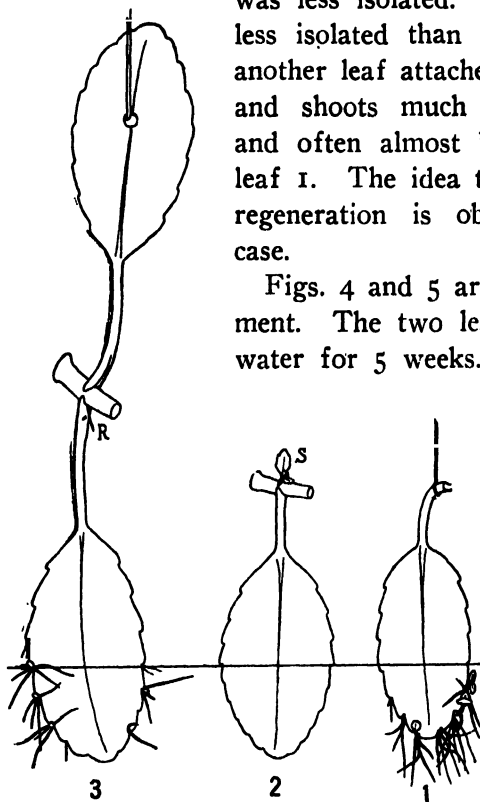
It is generally stated that "isolation" is responsible for regeneration, inasmuch as isolation would release the leaf from the inhibiting influence which the whole has on each part.<sup>1</sup> It is obvious, however, that isolation is an abstract term and that it cannot help us, therefore, in visualizing the forces inhibiting the growth of the buds while the plant is intact. We will show in a simple example that the conception of isolation, while it may fit some cases, will not fit others.

The following experiment was often repeated during the winter months. Three leaves of the same plant of *Bryophyllum* were suspended in an aquarium saturated with water vapor, so that the tips of the leaves (or about one-half of each leaf) were submersed in water at the bottom of the aquarium (figs. 1, 2, 3). Leaf 1 was completely isolated from the stem; leaf 2 had a piece of a stem of the plant attached; and leaf 3 had in addition to a piece of the stem of the plant also the opposite leaf attached. The drawings show the condition of the three leaves after 11 days. Leaf 1 formed roots in a few days, and soon after shoots at the notches of the submersed part of the leaf. In leaf 2, as a rule, all growth from the notches was inhibited, but the bud of the stem opposite the leaf grew out very rapidly into a shoot (fig. 2, S). The submersed part of leaf 3 again formed roots and stems in its notches, not quite but almost as quickly as leaf 1. Experiments showed that the result is the same if both leaves of specimen 3 are partly submersed in water; both form roots and shoots in that case.

According to the idea that isolation is the cause of regeneration, we should say that leaf 1 formed new roots and shoots because it was completely isolated; that leaf 2 did not do so (for a long

<sup>1</sup> CHILD, C. M., Die physiologische Isolation von Teilen des Organismus, etc. Roux's Vorträge und Aufsätze. Leipzig, 1911.

time at least) because, being connected with a piece of a stem, it was less isolated. But leaf 3, which was still less isolated than leaf 2, inasmuch as it had another leaf attached to the stem, formed roots and shoots much more quickly than leaf 2 and often almost but not quite as quickly as leaf 1. The idea that isolation is the cause of regeneration is obviously inadequate in this case.



FIGS. 1-3.

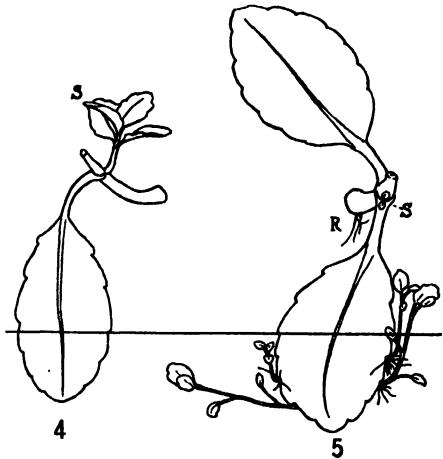
Figs. 4 and 5 are a repetition of this experiment. The two leaves had been submersed in water for 5 weeks. The leaf in fig. 4, with a piece of stem attached, had formed no roots or shoots in its notches; instead it had formed a long shoot (*S*) from the bud of the stem opposite the leaf. The leaf in fig. 5, with a piece of stem and the opposite leaf, had formed four shoots from the submersed notches, while the stem had formed one tiny shoot (*S*) from a bud in the axilla of the lower

leaf, and roots (*R*) at the under side of the basal end of the stem.

When we modify this experiment and suspend the three leaves entirely in moist air (instead of submersing them partly in water), leaf 1 (entirely isolated) will again form roots and shoots in its notches; leaf 2 will as a rule show no growth, but from the opposite bud of its stem a shoot will grow (*S* in figs. 2 and 4); and in the leaves 3 tiny roots may begin to grow from the notches which, however, usually dry up after some time; and no shoots are formed if the leaf is suspended entirely in moist air.

### III. *Inhibition of Growth of Leaves by Growth of Buds on Stem.*

The question now arises: Why does the presence of the piece of main stem in fig. 2 inhibit or retard the formation of roots and shoots in the notches of the leaf, and why does the same piece of stem cease to inhibit (or why does it inhibit considerably less) when, as in fig. 3, in addition to the stem another leaf is left with it? Each node has two buds, one in the axilla of each leaf. When we use a specimen, as fig. 2, a shoot (*S*) will grow out in a few days from that bud of the stem where the leaf is removed; and this is the first growth which will occur in this specimen. The bud in the axilla of the leaf which is preserved will as a rule not grow out. In fig. 3, where both leaves are preserved, neither bud of the stem will grow out in winter.<sup>2</sup> Hence we notice that where a shoot grows out very rapidly from the bud of the stem, as in fig. 2, the leaf in contact with the stem is prevented or delayed in forming roots and shoots, but when no such shoots grow out from the bud of the stem (as in fig. 3), the notches of the leaf (if submersed in water) will form roots and shoots rather quickly.



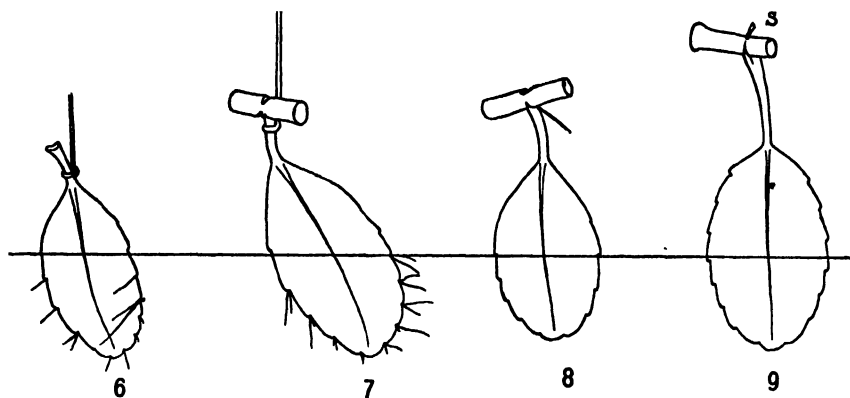
FIGS. 4 AND 5.

The stalk of an isolated leaf without any piece of the stem is not capable of giving rise to any regeneration. Such a leaf will form adventitious roots and shoots in its notches very rapidly.

All these facts make it appear as if the growth of the buds on a piece of the stem might have an inhibiting influence on the growth of the adventitious roots or shoots of the leaf.

<sup>2</sup> In the spring this is not so strictly true, but all these experiments were made in a greenhouse during the winter months. The greenhouse had a temperature of 70° F. or above.

In order to estimate properly such an influence, an extensive series of experiments was made, in which leaves with a piece of stem attached were submersed with their tips in water, while the rest of the specimen was in moist air. In a number of stems both buds were removed (fig. 7), in another only the upper buds were removed (fig. 8), while in the rest of the stems none of the buds were removed (fig. 9). If the inhibiting effect of the stem were



FIGS. 6-9.

*exclusively* due to the growth of the buds on the stem, the latter should lose its inhibiting effect entirely if these buds were removed; and the leaf connected with such a "debudded" stem should form adventitious roots or shoots as fast as a leaf without any stem. This was, however, not entirely the case. While a leaf connected with a "debudded" stem (fig. 7) formed as a rule its adventitious roots more quickly than a leaf with a normal stem (fig. 9), the leaves connected with the "debudded" stem formed their adventitious roots not quite so quickly as the completely isolated leaves (fig. 6).

Figs. 6-9 give the average results of such experiments. The drawings were made May 10, seven days after the operation. The isolated leaves (fig. 6) had all formed their adventitious roots, and so had some but not all the leaves with "debudded" stems (fig. 7). The leaves which had lost only the upper bud had not formed roots as fast as the leaves with entirely "debudded" stems (fig. 8). The leaves with normal stems (fig. 9) had not yet formed

any adventitious roots, but the shoot (*S*) on the stem where the leaf had been removed had begun to grow out.

The following record of an experiment performed May 1 had yielded on May 10 the following result:

1. Completely isolated leaves (fig. 6). All ten leaves had formed adventitious roots and tiny shoots.

2. Eighteen leaves each attached to a completely "debudded" stem (fig. 7). Eleven leaves had formed adventitious roots and one also adventitious shoots.

3. Ten leaves with a stem whose upper bud was removed (fig. 8). Four leaves had formed adventitious roots or shoots.

4. Ten leaves with a normal stem (fig. 9). All these stems formed shoots from the upper bud. No leaf has formed adventitious roots or shoots.

*It is, therefore, obvious first that a stem whose buds are removed has still an inhibiting influence upon the formation of roots in the notches of a leaf; and second, that if the buds of the stem are not removed the growth of the bud opposite the leaf enhances this inhibiting effect of the stem upon the leaf considerably.*

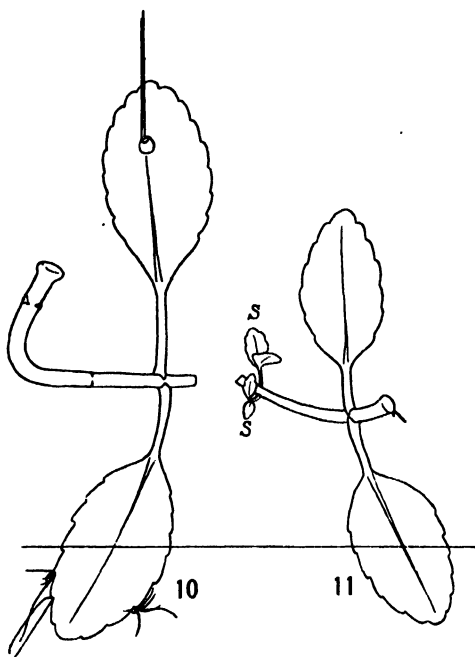
Since the growth of this bud of the stem is as a rule also inhibited when the opposite leaf is not removed, as in fig. 3, we understand why the non-removal of this leaf favors the growth of the adventitious roots from the notches of the other leaf.

We have seen that isolated leaves when suspended *in moist air* will form roots and shoots from their notches even if they are not submersed in water; while if a leaf is connected with a stem, the formation of roots and shoots in the notches will be permanently inhibited *in moist air*. It should be added, that the leaves attached to a "debudded" stem may form very short adventitious roots when suspended in moist air (instead of in water), but will not form long roots or shoots as will the completely isolated leaf. The analogy between the effect of the non-removal of the opposite leaf from the stem and of the removal of the opposite bud seems thus pretty complete.



#### IV. Continuation of These Experiments.

We have thus seen that the growth of buds on the stem is one factor which inhibits or delays the growth of the notches in the opposite leaf. We intend to show the influence of this factor in some further observations.



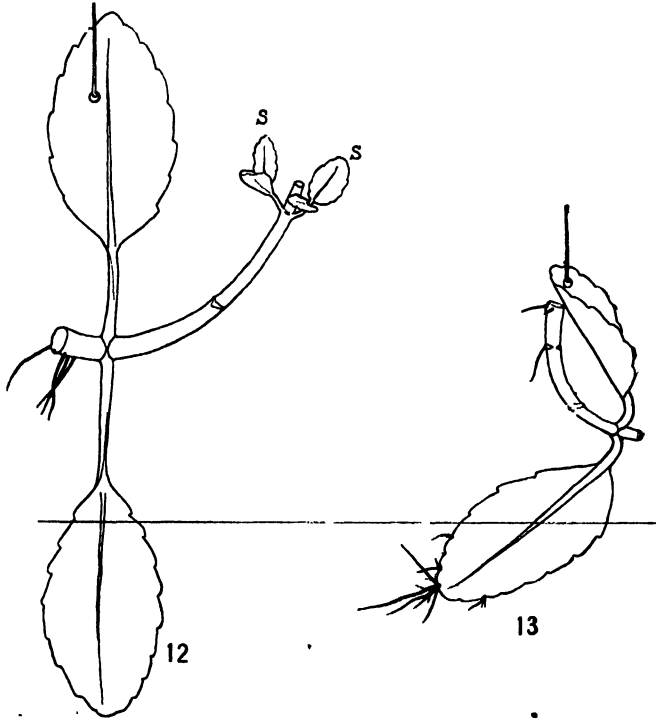
FIGS. 10 AND 11.

In all previous experiments we had cut out from a plant a piece of stem with only one node. If we cut out a piece of a stem containing two or three nodes (figs. 10, 11, 12, 13) and preserve one pair of leaves, the behavior of these leaves will be different if they are left in the apical or in the basal node of the piece. Figs. 10-13 illustrate this difference. In all cases one or both leaves are partly submersed in water, while the rest of the preparation

is suspended in moist air. In such cases new shoots (SS) were formed in a few days from the two apical buds of the stem in fig. 11, where the apical leaves had been removed and only the basal leaves left; while in specimens like fig. 10, where the apical leaves were left, the buds on the stem either formed no new shoots or formed them with some delay. As a consequence, we notice that in fig. 11 the submersed leaf formed at first no shoots, while the submersed leaf in fig. 10 in about 50 per cent of the cases formed roots and shoots in its notches rather rapidly. This happened often, but not always, when the formation of shoots on the stem itself was long delayed. Ultimately all the leaves may form adventitious roots and shoots in the notches that are under water or near the edge of the water.

This experiment, therefore, supports the conclusion that if the buds of the stem grow out very rapidly, their growth inhibits or delays the growth of roots and shoots in the notches of the leaf attached to the stem.

Figs. 12 and 13 are a repetition of the same experiment. The drawing was made 17 days after the beginning of the experiment.

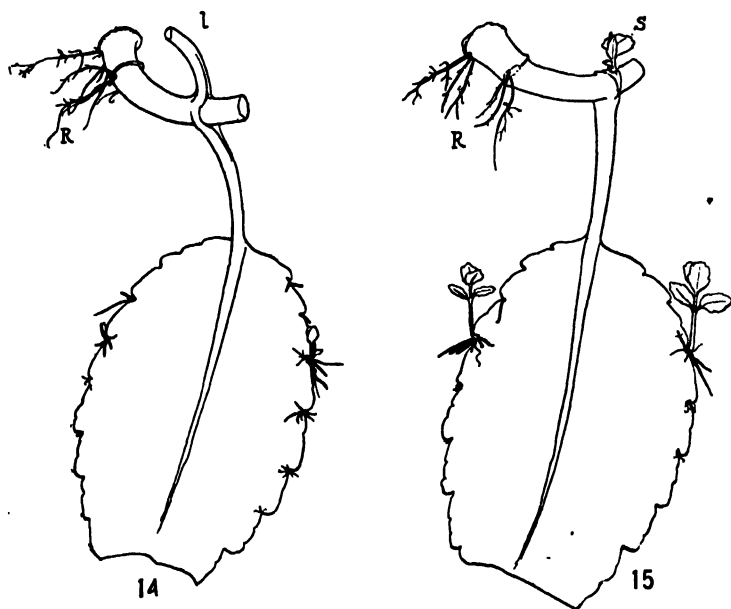


FIGS. 12 AND 13.

The stem in fig. 12 formed rapidly two shoots (SS) from its apical buds and this inhibited the growth of roots and shoots in the submersed leaf; in fig. 13 the stem formed no shoots and the submersed leaf could form roots. The root formation in both stems was about equal. These experiments have been repeated so often that they can be asserted to form reliable demonstration experiments, during the winter months at least.

We have already stated that in a completely isolated leaf (as in

fig. 1) the roots in the notches in the leaf do not begin to grow until a few days after the bud in a stem (*S* in fig. 2) has begun to grow. It would seem, therefore, that we might weaken the inhibiting influence of a piece of stem, as shown in the experiment in fig. 2, if we inhibit or retard the shoot formation of the bud on the stem. This can be done, as we have already stated, by not removing the other leaf on the stem, as in fig. 3. It is not necessary, however, to



FIGS. 14 AND 15.

leave the whole leaf attached to the stem; it suffices if we leave a piece of the stalk of a leaf attached as in fig. 14. In this case a leaf (with a piece of stem and a piece of the stalk *l* of the other leaf) were suspended November 12 in moist air. The bud in the axilla of the stalk *l* was by the presence of the latter prevented from growing out, and after some time roots (*R*) were formed at the basal end of the stem. Still later, roots and shoots began to grow out from the notches of the leaf (although this was not submersed in water). Fig. 14 was drawn January 18, therefore 9 weeks after the beginning of the experiment. About a week after the drawing

was made, the stalk *l* which had wilted fell off and now the bud in the axilla of the stalk *l* was able to grow and the shoot *S* was formed (fig. 14). The drawing (Fig. 15) was made when the shoot was one week old. The experiment shows also incidentally that the root formation on the stem does not (under the conditions of this experiment) inhibit the formation of roots and shoots on the leaf. We shall return to this fact later.

In this experiment the leaf was merely suspended in moist air and yet shoots developed from the leaf although it was attached to a piece of stem. This is unusual, since in order to obtain such a result with certainty it is necessary to submerge part of the leaf in water.

#### V. *Inhibiting Influence of Roots on the Growth of the Notches of a Leaf.*

A piece of stem when cut from a whole plant of *Bryophyllum* is not only able to form shoots but it also forms roots, and it is now our intention to consider the influence which the root formation of the stem has on the growth of the notches of a leaf. WAKKER, DEVRIES,<sup>3</sup> and GOEBEL<sup>4</sup> all have reached the conclusion that it is the presence of the main root or the regenerated roots on the stem which prevents the growth of adventitious roots or shoots on the leaf. If we break or cut off a leaf of *Bryophyllum calycinum* from the stem, neither the stalk nor the base of the isolated leaf has the power of forming roots, and this inability of root formation is considered by WAKKER and DEVRIES to be the cause of the growth of the notches. "According to WAKKER the organic separation of the leaf from the rooted part of the plant acts as a stimulus upon the leaf and induces the growth in the notches."<sup>5</sup>

DEVRIES describes a very striking experiment which supports the idea of WAKKER that the root of the main plant is the factor which inhibits the growth of the notches in the normal plant.

<sup>3</sup> DEVRIES, HUGO, Jahrb. Wiss. Bot. 22: 35. 1890.

<sup>4</sup> GOEBEL, K., Einführung in die Morphologie der Pflanzen. Leipzig, 1908 (pp. 142-149).

<sup>5</sup> DEVRIES, HUGO, *loc. cit.* The writer was not able to obtain WAKKER's monograph.

The apices of six plants were cut off beneath the most vigorous adult leaf and planted in soil. After strong roots had been formed (in the soil) their stems were cut above the lowest pair of leaves, the apices removed, and this lowest pair selected for the experiment. Both leaves were put flatly on moist sand, the one after having been removed from the stem, while the other remained connected with the roots. The axillary buds were destroyed. After three weeks the isolated leaves had formed numerous young plants on their margin. The leaves which had remained connected with the rooted piece of stem had formed no plants in their notches (and did not form any afterward), although they had otherwise been exposed to the same conditions as the isolated leaves.

This experiment is in harmony with the view that the normal roots of a stem (if they are under normal conditions) inhibit the growth of notches in the leaves. DEVRIES reports a second experiment in favor of the view of WAKKER. He cut the stem of a plant in its internodes and thus isolated seven pairs of leaves.

From each pair one leaf was broken off; all axillary buds were destroyed. The leaves were now put on moist sand. After a month the seven stems had formed roots. The isolated leaves<sup>6</sup> had formed in their notches rooted plantlets varying from 10 to 26 in number. The leaves whose stems had formed roots behaved differently. One leaf had formed no trace of growth in its notches; it was the one whose stem had formed roots first. The rest of the leaves had formed only a few plants whose number varied between 2 and 6. They reached only a few mm. in length, while those of the isolated leaves measured from 0.5 to 2 cm.

We see here that if a root is formed on a stem before the roots in the notches of a leaf can grow out, the root (under proper conditions) may inhibit the growth of the notches of a leaf.

While these facts leave no doubt that the root (under proper conditions) can inhibit the growth of the notches in the leaves of *Bryophyllum*, the experiments mentioned on the previous pages of this paper show that this is not the only factor. A piece of stem, even if it does not form any roots but only a shoot, will inhibit or greatly delay the growth of the notches of a leaf connected with it.

#### *VI. Influence of Root Formation and of Root Pressure.*

DEVRIES assumes with WAKKER that it is not the root formation in itself by which the stem or main plant inhibits the growth

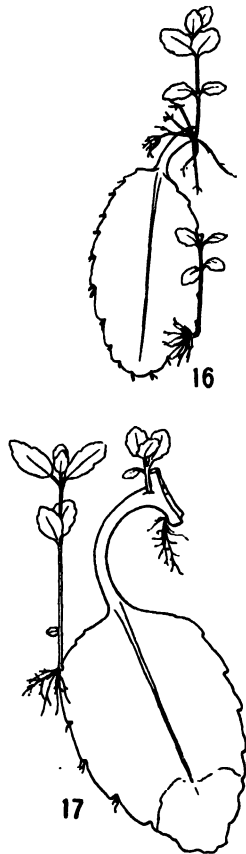
<sup>6</sup> That is, those broken off from the stem.

in the notches of a leaf, but the root pressure. GOEBEL is inclined to think that it is the root formation in itself, regardless of the root pressure (or flow of water caused by it), which inhibits the growth in the notches of the leaf. The writer has made a number of observations which indicate that of the two views that of WAKKER and DEVRIES is better supported by the facts.

As an illustration, we may take figs. 14 and 15, in which a leaf with a piece of stem was suspended in moist air. The basal end of the stem formed a mass of roots, yet this did not prevent the growth of roots and shoots from the notches of the leaf. This contradicts GOEBEL'S assumption, but is in harmony with the view of DEVRIES, since these roots in the air were not able to give rise to "root pressure."

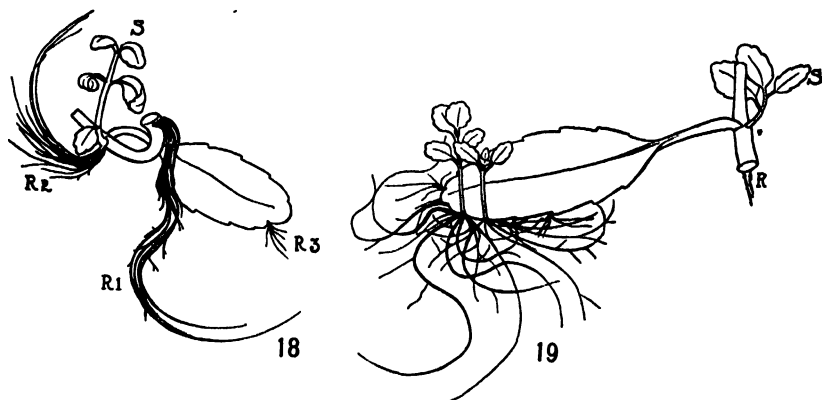
As a further support, we may give the drawings (figs. 16 and 17). In these cases the leaves were cut off with only a fragment of the stem attached, which was a little larger in fig. 17 than in fig. 16. The axillary bud was not removed. The leaves were suspended in moist air. Although the remnants of the stems formed roots, yet the leaves formed also roots and shoots (although they were in moist air). The root formation on the remnant of the stem preceded the root formation on the leaf but did not prevent the latter. The experiment shows again that mere root formation in a stem suspended in moist air does not prevent the formation of roots and shoots in a leaf of *Bryophyllum*. It should be pointed out, however, that the shoots grew out from the axillary bud of the leaf; it is the growth of the opposite bud which has the inhibitory power on the leaf mentioned in the third and fourth sections.

While the root formation on the stem does not inhibit the shoot formation on the leaf if the root is exposed to moist air, the result



FIGS. 16 AND 17.

is different if the roots are in water. If one leaf with a piece of stem (fig. 18) is put into a Petri dish, the bottom of which contains a thin layer of water, the stem will form enormous roots ( $R_1$ ) at its basal end from the callus, and  $R_2$  from the basal end of the shoot ( $S$ ) which grows out from the bud of the stem where the leaf was removed. On the other hand, the leaf has formed only a few small roots ( $R_3$ ) at one notch. (As a rule the notches of the leaf formed no roots in such an experiment.) In this case the roots of the stem which were functioning, and probably established the



FIGS. 18 AND 19.

usual root pressure, inhibited for a long time and in most cases permanently the regeneration in the leaf.

If, however, we do not put the leaf immediately after it is cut from the plant into the Petri dish but suspend it first in moist air, the stem will form a shoot ( $S$  in fig. 19). Roots ( $R$ ) may or may not be formed on the stem, but they will always be formed considerably later than the shoot, at least in winter. If after a month we put the leaf into a Petri dish, while the stem remains in moist air, the leaf (fig. 19) will rapidly form roots and shoots. The contrast between the behavior of the leaves in this case and the one mentioned before is very striking. In the experiment represented in fig. 19, the roots ( $R$ ) at the base of the stem could not establish a flow of water in the stem and could not inhibit the growth of the shoots in the notches of the leaf; and by the time the leaf was

put in water they were obviously not in a position to produce a flow or a root pressure.

Roots formed on the stem have as a rule, therefore, an inhibiting effect on the growth from a leaf if they can produce a root pressure, that is, if they are in water. The formation of a shoot from the bud of a stem can produce such an inhibiting effect upon the opposite leaf if the shoot is in moist air and if no root is formed. This influence of roots on the growth of the notches of a leaf was discussed only for the sake of completeness, since it does not strictly belong to our problem, which deals only with the growth of shoots.

### *VII. The Conditions Inhibiting and Accelerating the Growth of the Axillary Buds.*

Each node of *Bryophyllum* has one pair of leaves, and in the axilla of each leaf is found a bud which in the normal life never grows out, but which may grow out as a consequence of a mutilation of the plant.

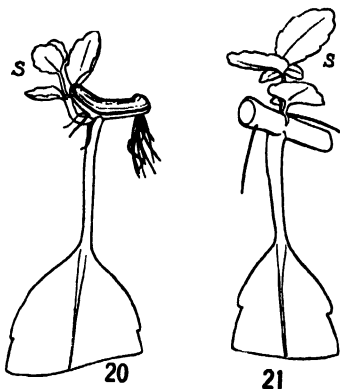
If we cut through two successive internodes of a stem and isolate a single node, and if we remove the two leaves, the two buds on the stem will grow out rapidly (if we provide the necessary water supply or if the node was cut out from near the base of the stem).

If we remove only one instead of both leaves, only one bud will as a rule begin to grow, namely the one whose leaf is removed. This suggests the idea that the leaf, while favoring the growth of the opposite bud, inhibits the growth of its own axillary bud. If we remove neither of the two leaves, in many cases (especially in winter) neither bud will grow out, a fact which harmonizes with the assumption that each leaf suppresses the growth of its own axillary bud.

The following experiment, however, restricts this last assumption that each leaf will inhibit the growth of its axillary bud. If we isolate a node with its two leaves (which we do *not* remove), and if we split the piece of stem longitudinally, we obtain two leaves, each attached to a half of a node containing the axillary bud of the leaf (fig. 20). In this case the axillary bud will grow out, although often with some delay. Hence the leaf in this case does



not prevent the growth of its own axillary bud, and if we speak of an inhibition in the previously mentioned cases, we have to add the remark that this inhibition only exists if the other leaf or the opposite bud are in connection with the first leaf. A comparison of figs. 20 and 21 is of interest. In both cases leaves with a piece of stem attached were suspended in moist air on February 20. They were drawn on April 1. In fig. 20 the shoot (S) in the axilla of the leaf left attached to a longitudinally split piece of stem grew out. In fig. 21, one leaf with a whole, non-split piece of stem attached gave rise to the growth of the shoot (S) on the upper side of the stem where the leaf was removed, while the bud in the axilla of the leaf was prevented from growing.



FIGS. 20-22.

Fig. 22 is a case similar to fig. 14, one leaf with a piece of stem and the stalk *l* of the opposite leaf. In this case the bud (S) in the axilla of the intact leaf grew out. This is not the most common experience. More often in winter neither of the two axillary buds of the stem will grow out under such conditions. The experiment in which the piece of stem is split longitudinally (fig. 20), however, generally succeeds.

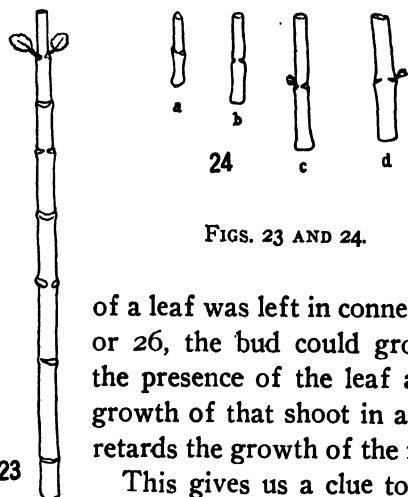
The following observation is also of some significance. If we cut out a node, remove one leaf and its bud, but preserve one leaf and the bud in its axilla, the latter will grow out into a shoot after some delay. Hence the removal of the opposite bud removes the inhibiting influences which this bud naturally has on the growth of the bud in the axilla of a leaf. We can accelerate the growth of this latter bud, however, when in addition to the removal of the opposite bud and leaf we make an incision or cut out a piece from the rind apically to the axillary bud whose leaf is not removed. In this case the bud in the axilla of a leaf which is not removed will grow out rather rapidly.

We may anticipate that all these experiments indicate that the growth of the bud depends upon the flow of certain substances from the leaf to the bud. That bud which receives these substances first will grow out first, and thereby prevent the flow to the other bud whose growth is thereby "inhibited." The apparent inhibition of growth in one place is simply due to the fact that under the conditions of the experiment the substances required for growth flow to some other place and are retained there, and the removal of the inhibition consists in creating conditions which will force the substances to flow where we want growth to occur.

#### *VIII. The Rules and Mechanism of Inhibition in Regeneration.*

We cut off the base and tip of the main stem of a plant of *Bryophyllum*, remove all the leaves, and suspend the stem in a closed aquarium saturated with water vapor. Only the two buds at the highest apical node will grow out (fig. 23); it does not matter whether the stem is hung upright or inverted. The buds at the more basal nodes are all inhibited from growing by the growth of the two apical buds; for if we isolate any of the lower nodes, their buds also may grow (fig. 24). This is the well known example of an inhibition of one part by another. In the terminology of REINKE, we might call the two apical buds the "dominants." What is the source of their dominance? By way of an answer we intend to show that the following relation exists: *If an element a inhibits the growth in an element b, b very often accelerates or makes possible the growth in a.* When we cut off a single node near the top of the main stem of *Bryophyllum*, remove the two leaves, and suspend it in an aquarium saturated with water vapor, as a rule the two buds will not grow out. If, however, we leave it in connection with one or more of the lower nodes of the stem, it will regenerate, and incidentally inhibit the growth in the lower nodes (figs. 23, 24). The regeneration and growth of the two shoots at the apical node will as a rule be the quicker the more nodes are left in contact with it. Hence the lower part of the stem whose regeneration is inhibited by the apical node, at the same time accelerates the latter's regeneration or makes it possible.

The second example is the following: When we cut off one leaf with a piece of the main stem (as in fig. 2) and suspend it in water, the bud opposite the intact leaf will grow out into a shoot (*S* in fig. 2). We have seen that the growth of this shoot has a share in the inhibition of the growth of the notches of the leaf in this experiment. It can be shown that conversely the leaf accelerates or renders possible the growth of the bud in the stem. As stated, the isolated node near the top will not be able to form shoots if suspended in moist air.



FIGS. 23 AND 24.

If, however, one leaf or even a fraction of one leaf is left in connection with the stem, the bud on the opposite side will grow out (figs. 25–29). In the isolated nodes (figs. 28, 29) cut off near the apex no buds could grow in moist air.

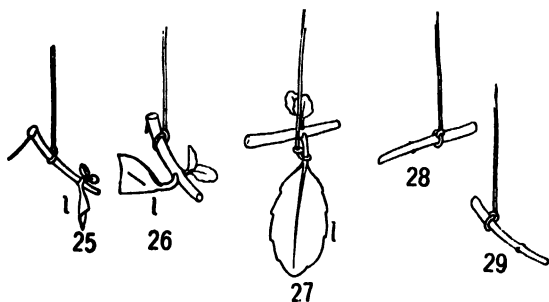
When, however, only a piece of a leaf was left in connection with such a stem, as in fig. 25 or 26, the bud could grow out. Here we see again that the presence of the leaf accelerates or renders possible the growth of that shoot in a stem whose formation inhibits or retards the growth of the notches of the leaf.

This gives us a clue to the nature of the dominance and the power of inhibition. The inhibition seems to consist in this, that the dominant part receives something from the inhibited part which accelerates growth or renders it possible in the former.

When we put an isolated node from near the top (whose leaves are removed and which cannot regenerate in moist air) in a very thin layer of water, new shoots grow out (figs. 30–32). This looks as if the “something” which the inhibited part supplies to the dominant part were water. But the writer is suspicious that the water may be only indirectly needed, namely to render the flow of material in the conducting vessels possible. In animals we know that the blood vessels must be filled to render a closed circulation possible. It would seem as if in plants a flow of substance through conducting vessels should be possible only if a certain minimum amount of water is contained in the conducting cells or vessels.

The buds of an isolated node nearer the base of the stem may grow out if suspended in moist air, probably because such a piece does not dry out so easily.

The following experiment rarely fails. If we suspend a piece of stem consisting of several nodes and stripped of all leaves in moist air (fig. 33), the two most apical buds (*b*) will grow out. Their growth, which is usually slow, is greatly accelerated if we leave one leaf (or more) on the stem (*b* in fig. 34). In two weeks the growth of the apical buds (*b*) in fig. 33, which had no leaves, was very slight, while it was strong in the stem (fig. 34) in which one



FIGS. 25-29.

leaf was left. Here we have the combined accelerating effect of stem and leaf upon the growth of the apical bud.

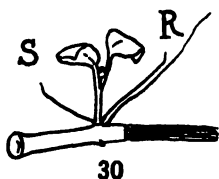
Why is it that the apical bud grows out first? Should this be connected with the anatomy of the conducting vessels, possibly in the way that the majority of these vessels go directly from the leaves to the growing point at the apex?

Since the rapid growth of the bud on a stem inhibits or retards the growth of adventitious roots of the opposite leaf (fig. 2), it follows that the removal of the bud or the inhibition of its growth should favor the growth of adventitious roots in the notches of the leaf. This is indeed the case. If we suppress the growth of the two buds in an isolated node, we favor the growth of adventitious roots in the leaves if they are submersed in water (fig. 3). The same happens if we split the node longitudinally (figs. 35, 36). The leaf (fig. 35) connected with a longitudinal half of a node was submersed in water and formed adventitious roots in nine days. The leaf

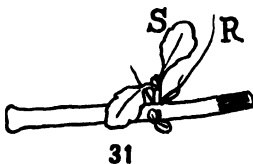
(fig. 36) attached to a whole node formed no adventitious roots under the same conditions.

### *IX. Isolation, Inhibition, and the Flow of Material through the Plant.*

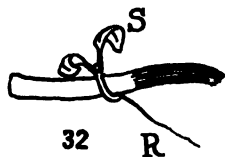
These rules give us some basis on which we may try to form a preliminary idea on the nature of the mechanism of inhibition.



As we mentioned already, the rules are comprehensible if we assume a flow of certain (possibly specific) substances (or formed cells) from the places where the dormant buds are ready to grow, or the prevention of such a flow toward these dormant buds.



We will first show in a few simple examples that this idea leads us easily through the maze of facts in which the terms isolation or inhibition have no more than a metaphorical value.



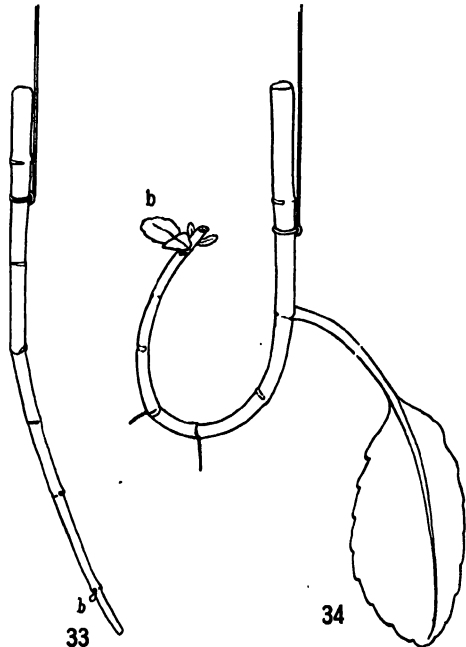
FIGS. 30-32.

When we isolate a leaf and suspend it in moist air or put it into a Petri dish the bottom of which is covered with water, as a rule only a few of the notches will grow out into shoots. Why do not all grow out? From what was said in the previous section it was natural to expect that the growth of the shoots in some of the notches of a leaf inhibits the growth in the rest of the notches of the

same leaf, and that if all the notches could be isolated from each other this inhibiting effect would cease and they would all grow out. This idea was put to a test in a way indicated in fig. 37. Five notches on one side of a leaf were isolated from the leaf and from each other. The rest of the leaf and the isolated notches were put into a Petri dish whose bottom was covered with a layer of water. All five isolated notches grew out into shoots, while only three of the ten or twelve notches left on the leaf grew out. This experiment, which has often been repeated, succeeds easily if the

piece of leaf left around each notch is not too small. It is noticeable that the rapidity of growth is greater in shoots which grow out from a whole leaf than in those growing out from the isolated notches. Here we see again an application of the rule that if an organ *a* inhibits the growth in *b*, the presence of *b* accelerates the growth in *a*. This is intelligible on the assumption that the leaf furnishes a flow of liquid containing material for the growth of shoots; and that the flow of this material away from the notches (wherever this may be) leads to the inhibition of the growth in the notches.

When the piece of leaf around an isolated notch is too small, no growth may occur or only tiny roots or shoots will grow out. This observation again agrees with the assumption that a notch of a leaf will grow into roots and shoots if certain substances or formed constituents of the leaf flow toward a notch or are prevented from flowing away.

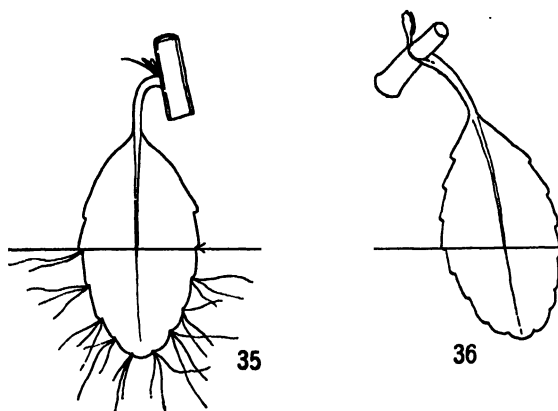


FIGS. 33 AND 34.

We can understand the experiment illustrated in fig. 37 on the assumption that if in a leaf one or more notches begin to grow out into roots and shoots, these shoots determine a flow in the rest of the leaf in a similar way as if a piece of the stem had remained attached to the leaf; and with the same inhibitory or retarding effect upon the growth of the other notches of the leaf. If, however, each notch is isolated and given enough water (for example, if it is put into a Petri dish which has a very thin layer of water), each notch can grow out, since the in-

hibitants through the establishment of currents in the leaf to growing shoots are lacking.

We have stated that if a leaf is suspended in moist air the growth of the shoots is prevented if a piece of a stem is left attached to the leaf. It seemed of interest to find out if this inhibiting effect would show itself even in a leaf in which a number of lateral incisions were made. This is indeed the case, as figs. 38 and 39 show. In these experiments the incisions were such that the pieces of the leaf had to be kept together by stitches of a thread. The leaves were suspended in moist air. Yet complete inhibition of the growth of



FIGS. 35 AND 36.

the notches occurred in the leaf which was connected with a piece of stem (fig. 38). If a flow of substances from the leaf to the piece of stem is the cause of inhibition, such a flow must have taken place along a zigzag path in the leaf. One finds occasionally in such an experiment that in the extreme apical piece of the leaf the inhibiting effect of the stem may cease and that there a growth of roots may occur in the notches.

That the flow of water and of the material it carries in a stem may be deviated and altered by the growth of new shoots is rendered obvious by such observations as are represented in figs. 40 and 41. In this and similar cases thick pieces of the stem of *Bryophyllum* were cut out from the plant, deprived of their leaves, and put on

moist soil. As is usual, shoots grew out very soon from the top buds of the stem; very soon afterward the piece in front of the top node began to shrink and wilt, not directly to the top node, but to within a few millimeters (fig. 40). When by chance the new shoot grows out not from the top node but from the one next to it, the whole piece in front of the top node may wilt (fig. 41).

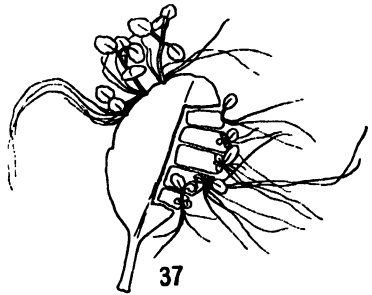
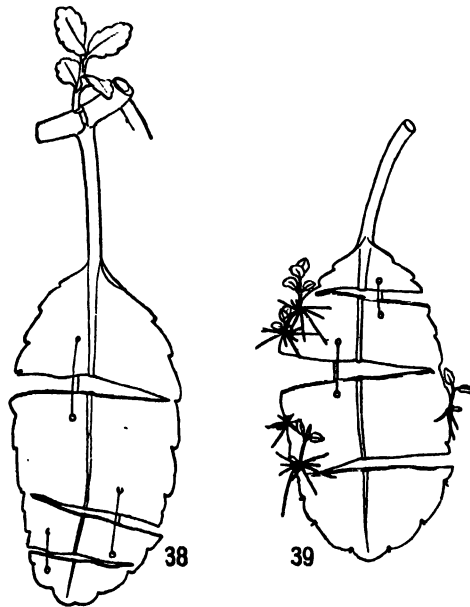


FIG. 37.



FIGS. 38 AND 39.

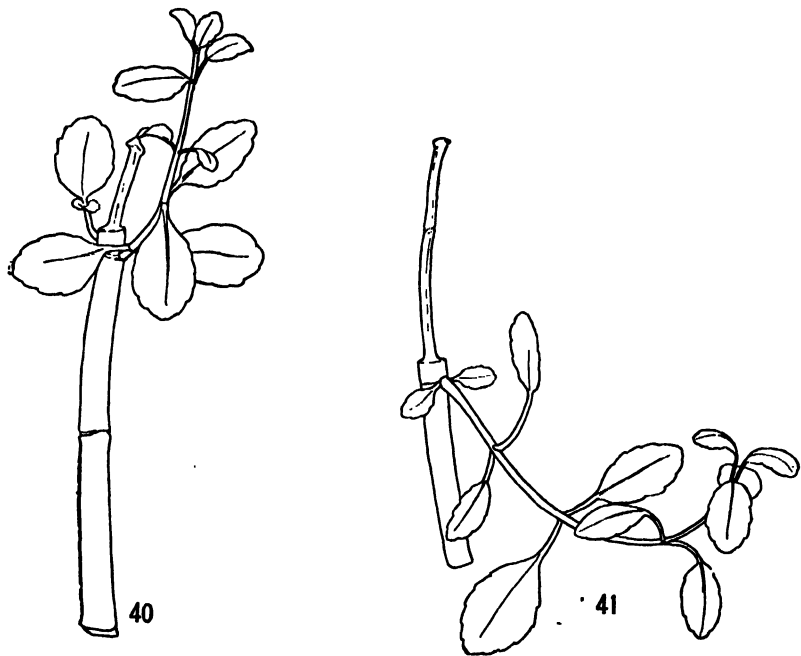
this wilting of the piece of the stem in front of the node from which the new shoots grow out will not occur. The ascending flow of liquid or material in the stem was deflected in this experiment into the most apical bud, and there was not enough root pressure to maintain a flow through the pieces of the old stem more apical than the new shoot.

By way of parenthesis we may here briefly mention that light exercises a great influence on the growth of the notches of an isolated leaf. If such

leaves are suspended in moist air but free from light, as a rule none of the notches will grow out, while they will grow out promptly as soon as they are exposed to the light; provided they had not



been kept too long in the dark. If the leaves are kept in the dark in a Petri dish whose bottom is covered with water, a few notches may grow out, but they are not nearly as numerous as those growing out in the light. Whether we are dealing here with a direct chemical effect of the light or with an indirect effect on the flow of substances



FIGS. 40 AND 41.

in the leaf remains to be seen. It should not be overlooked that as soon as the leaves of *Bryophyllum* turn yellow they become less turgid and easily fall off from the stem.

#### *X. Theoretical Remarks.*

We may now go back to the first experiments mentioned in this paper and try to analyze them on the basis of the old idea that the flow of material in the plant is responsible for phenomena of growth. We start from the assumption that a notch of a leaf can grow out only if there is no flow of liquids (carrying non-formed or possibly formed material) away from the notches. This is not

the case in the normal plant when the circulation is normal, and WAKKER and DEVRIES have shown the rôle which the root pressure plays in this case. But the root pressure is not the only factor which influences this flow. The experiments in figs. 1-3 seem to indicate that different factors aside from the root pressure can determine the flow away from the notches of the leaf, provided our assumption is correct that such a flow is the cause of the phenomena of growth and regeneration observed.

If we go back to these first experiments in this paper and try to formulate them in harmony with this idea, we should have to state that in a completely isolated leaf the flow away from the notches ceases. As a consequence, one or more of the notches may grow out, and as soon as this happens the flow in the leaf is directed toward the growing notches. They act as if they exercised a "suction" on the flow of liquids in the leaf, and they may inhibit the growth in other notches of the leaf.

If the leaf is in connection with a piece of stem, the latter exercises this "suction," and the flow of liquids is away from the leaf to the stem; hence the inhibiting effect of the stem upon the growth of the notches of the leaf. This "suction effect" is especially great if the bud opposite this leaf can grow out, as in fig. 2. If both leaves are left attached to the piece of stem (as in fig. 3), the flow from a leaf will be deflected from the buds and may go into the opposite leaf. This might explain why when both leaves remain attached to a piece of stem the growth of the notches of the leaves is favored again, though it is not so rapid as in a completely isolated leaf.

This idea of a deflection of the current away from the leaf toward the opposite side of the stem is in harmony with the fact that the bud opposite a leaf grows out very quickly if its own leaf is removed (fig. 2); while the growth of the axillary bud of the leaf which is not removed is inhibited in this case. If we split the stem longitudinally, this deflection ceases and the leaf ceases to inhibit the growth of its own axillary bud. This idea is supported by the fact that if the leaf attached to a longitudinally split node is partly suspended in water its notches will grow out rather rapidly.

(We have assumed that if we have a node with its two leaves attached, the flow will be deflected from the buds; this again is in

harmony with the facts that in such a case as a rule neither of the buds grows out.

When a plant is normal, it is almost or possibly absolutely impossible to induce the notches of a leaf which is connected with the plant to grow. The writer has submersed such leaves in water, but in months not a single notch ever formed a root or a shoot. If, however, the flow of substances in a plant is abnormal, either because the roots or the apical parts or both have suffered, a growth of shoots may occur in moist air from the notches of leaves which are in contact with the plant. This fact is mentioned by DEVRIES and is well known to those who have seen the plants in their natural conditions in Bermuda.

If we now return to the question from which we started, namely, why it is that the notches of the leaves of *Bryophyllum* will not grow out while in connection with the normal plant, the answer should be that the flow of material from the root and from the leaves into the stem and to the apical end of the latter prevents this growth. Through this flow material is carried away from the notches of the leaves. The anatomy of the conducting vessels and tissues, which is inherited, and the dynamical factors determining the flow are the factors concealed in the term "correlation." We understand why it is that if we isolate a part, buds may grow out which without the isolation would not have grown, the reason being that in the mutilated part material can flow to and be retained at places where if the part had remained in the whole it could not have been retained. This assumption agrees with the older ideas of DUTROCHET, SACHS, DEVRIES, and GOEBEL on regeneration in plants. We understand on this basis why it is that the term isolation of parts or the inhibiting effect of growing parts on others may express some but not all the facts of regeneration. It is not the isolation in itself, but the retention of material in places where there would not have been such a retention under ordinary conditions which apparently determines the growth of dormant buds in an isolated piece; and so it may happen that while this term expresses adequately some results, it fails in others.

SACHS assumed that specific organ-forming substances were needed for growth, and that the consumption of these substances in

the growing regions was the cause of the inhibition of growth in the dormant buds of a plant. While the first half of the theory may be correct, the second part is not tenable, since in each stem of a *Bryophyllum* there is enough "formative" material to allow each bud in all the nodes to grow out, while as a matter of fact only the most apical ones will do so. This is intelligible on the assumption that these apical nodes retain the "formative" material in excess of what they need for their own growth.

The ideas expressed in this paper agree in the main with the results and conclusion of the author's older experiments on regeneration in animals. The writer had found that if a piece be cut out from a stem of a *Tubularia* a new polyp may form at each end of the stem, but that the formation of the polyp at the oral end precedes that at the aboral end; and the difference in time may be from one or two days to as many weeks, according to the species or the temperature. He found also that the formation of the oral polyp is the cause of the delay in the formation of the aboral polyp, and that if he prevented the formation of the oral polyp this delay in the formation of the aboral polyp was no longer observed.<sup>7</sup> This is the same rule which we have found for the relation between the growth of the bud of the stem and the formation of adventitious roots and shoots in the opposite leaf of *Bryophyllum*. The growth of this bud causes a delay in the growth of adventitious roots and shoots in the opposite leaf, and this delay is suppressed or diminished if the bud is prevented from growing.

The writer suggested that a flow of substances was the cause of these phenomena of correlation in *Tubularia*. He had found that pigmented cells which come from the entoderm and are carried in the circulation are always collected at the spot where regeneration of the natural growth of the hydroid is to start.

These remarks may suffice to indicate that the rules of inhibition observed in *Bryophyllum* may have a wider application.

<sup>7</sup> LOEB, J., Untersuchungen zur physiologischen Morphologie der Tiere. I and II. Würzburg, 1890 and 1891; Pflüger's Archiv 102: 152. 1904.

## XI. SUMMARY OF RESULTS.

The phenomena of inhibition of regeneration have been studied in *Bryophyllum calycinum* and it was found that they are governed by the following simple rule:

If an organ *a* inhibits the regeneration or growth in an organ *b*, the organ *b* often accelerates and favors the regeneration in *a*.

This rule is best understood on the assumption that the inhibiting organ receives something from the inhibited organ necessary for regeneration.

It is pointed out that this harmonizes with the older assumption of botanists and of the writer that the flow of material and the block to such a flow after mutilation are responsible for the phenomena of inhibition in regeneration, as well as for the phenomena of correlation.

## THE INFLUENCE OF ELECTROLYTES UPON THE DIFFUSION OF POTASSIUM OUT OF THE CELL AND INTO THE CELL.

By JACQUES LOEB AND McKEEN CATTELL.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, September 2, 1915.)

### I. INTRODUCTION.

We intend to show in this paper that potassium cannot diffuse out of the egg of *Fundulus* when the latter is put into distilled water or into a saccharose solution, while potassium can diffuse out from the egg easily when the latter is put into a solution of an electrolyte; and that the influence of electrolytes upon this diffusion increases with the valency of the anion of the salt and follows possibly the Hofmeister series of anions. We will also show that when we keep eggs for some time in  $H_2O$  and put them afterwards into a KCl solution a barrier is created which retards the entrance of potassium into the egg. These experiments have a bearing not only upon the mechanism of a certain group of phenomena of antagonistic salt action but also upon the mechanism which determines the diffusion of cations through cell walls. Certain theoretical aspects of these experiments have already been discussed in a previous paper.<sup>1</sup>

Loeb and Wasteneys have shown in 1911<sup>2</sup> that the marine fish *Fundulus* is rapidly killed by a pure KCl solution in that concentration of this salt in which it is contained in sea water, namely 2.2 cc.  $M/2$  KCl in 100 cc.  $H_2O$ ; while the fish live indefinitely when NaCl is added in such a ratio that the solution contains 17 molecules or

<sup>1</sup> Loeb, J., *Proc. Nat. Acad. Sc.*, 1915, i, 473.

<sup>2</sup> Loeb, J., and Wasteneys, H., *Biochem. Ztschr.*, 1911, xxxi, 450; 1911, xxxii, 155.

more of NaCl for each molecule of KCl.  $\text{Na}_2\text{SO}_4$  was approximately twice as effective as NaCl but the quantitative determinations were restricted by the great toxicity of  $\text{Na}_2\text{SO}_4$ . Experiments on the possible antagonistic action of other salts than NaCl or  $\text{Na}_2\text{SO}_4$  could not well be made since most salts were too toxic for the fish. Loeb and Wasteneys explained this result on the assumption that the presence of NaCl or  $\text{Na}_2\text{SO}_4$  in the solution prevented the KCl from diffusing into the fish. Assuming that the KCl, in order to diffuse into the fish (*i.e.*, into the blood and thus reaching the heart and the central nervous system), had to undergo a combination with a colloidal constituent of the skin of the fish, the Na ions of the NaCl or  $\text{Na}_2\text{SO}_4$  by competing with the K for the colloidal anion would prevent the latter from combining with this constituent.

Our new experiments were made on the embryo of *Fundulus* instead of on the adult fish. The embryo is surrounded by the rather thick egg membrane inside of which it moves freely. Any salt, before it reaches the embryo, must therefore diffuse through the membrane. When we put an egg containing an embryo from four to fourteen days old into a pure KCl solution until the heart of the embryo stops beating we know that the KCl must have diffused through the egg membrane into the egg in such a quantity as to reach the toxic limit for the heart of the embryo; and if such an egg is afterwards put into a solution free from KCl, where its heart recovers, we may conclude that enough KCl must have diffused from the egg into the outside solution, so as to bring the concentration of KCl inside the egg below the limit required to cause the standstill of the heart.

## II. THE INFLUENCE OF THE ANION IN THE PREVENTION OF THE TOXIC ACTION OF KCl.

When we try to investigate the prevention of the toxic action of KCl on the adult fish by other electrolytes, we are restricted by the fact that only a few salts are sufficiently harmless for the fish to be used for such a purpose. In the case of the embryo, which is separated from the outside solution by the egg membrane, we have a much greater freedom in the choice of our antagonistic salts.

In the experiments to be mentioned in this chapter we ascertained the influence of the concentration of salts and the sign and nature of the ions upon the rapidity with which a given concentration of KCl caused the hearts of the embryos to stop beating. As a rule, twenty embryos of the same age whose hearts were beating were put into each solution and the number of embryos whose hearts were still beating was ascertained at various intervals.

We found first that if we add to a given concentration of KCl another salt, *e.g.*,  $\text{Na}_2\text{SO}_4$ , in various concentrations, the poisonous action of KCl upon the heart will be the more retarded the higher the concentration of  $\text{Na}_2\text{SO}_4$ . In one experiment of this kind 6.6 cc.  $\text{M}/2$  KCl were contained in 50 cc. of the solution.

TABLE I.

After	Number of surviving hearts in 6.6 cc. $\text{M}/2$ KCl dissolved in 50 cc. of						
	$\text{H}_2\text{O}$	$\text{M}/4$	$\text{M}/8$	$\text{M}/16$	$\text{M}/32$	$\text{M}/64$	$\text{M}/128$ $\text{Na}_2\text{SO}_4$
<i>hrs.</i>							
1	14	20	20	20	15	10	11
4	5	20	17	11	11	8	9
9½	3	19	16	7	4	3	2
24	1	19	13	4	3	3	0
72	1	14	9	2	1	2	0

While after nine and a half hours practically all the hearts in  $\text{M}/4$   $\text{Na}_2\text{SO}_4$  were still beating, in the solutions of  $\text{Na}_2\text{SO}_4$  below  $\text{M}/32$  and in distilled water only four or less of the original twenty were beating.

An experiment with Na acetate in different concentrations gave a similar result.

We next tried the effect of different sodium salts to find out whether the anion had any effect in this case. This was found to be true. 6.6 cc.  $\text{M}/2$  KCl were dissolved in 50 cc. of the following solutions (Table II). Twenty embryos, five days old, were put into each of the solutions.

The experiment shows that the antagonistic effect of  $\text{Na}_2\text{SO}_4$  and  $\text{Na}_2$  tartrate is very powerful since after two days the hearts of almost all the embryos were still beating. Next in efficiency was the acetate which was found to be much more effective than NaCl.



The antagonistic effect of a salt against KCl is therefore in this series a function of the anion and grows with the valency of the latter. The order of efficiency of the anions is:



This suggests to some extent the Hofmeister series of anion efficiency and this statement will find fuller corroboration in a later chapter. We come, therefore, to the conclusion that KCl causes the hearts of the embryo of *Fundulus* to stop beating most rapidly when alone in solution, while sodium salts inhibit or retard this effect, (a) the higher (within certain limits) their concentration, and (b) according to the nature of the anion of the salt added, the order of increasing efficiency being



It is desirable to take for these experiments young embryos, since in older embryos the time required for causing the standstill of the heart by KCl is greater.

TABLE II.

After	Number of hearts beating in 6.6 cc. M/2 KCl in 50 cc. of								
	H <sub>2</sub> O	M/4 sea water	M/4 NaCl	M/4 NaBr	M/4 NaI	M/4 Na acetate	M/4 NaCNS	M/4 Na <sub>2</sub> SO <sub>4</sub>	M/4 Na tartrate
hrs.									
1	11	19	18	18	18	20	16	20	20
4½	2	12	14	11	16	15	3	19	19
22	1	11	11	8	3	13	1	18	16
48	1	7	9	4	1	14	0	18	16

These experiments confirm the older observations of Loeb and Wasteneys on the adult fish, but they add the fact of the rôle of the anion in this antagonism. This rôle could not be ascertained on the adult fish since most of the salts, like tartrates and acetates, are too toxic for the adult fish to be used for antagonistic purposes.

We also confirmed the result found in the previous investigation,<sup>3</sup> that it was only possible to inhibit the poisonous action of KCl by other salts as long as the concentration of KCl did not exceed a certain limit.

<sup>3</sup> Loeb and Wasteneys, *Biochem. Ztschr.*, 1911, xxxi, 450.

### III. THE IMPOSSIBILITY OF RECOVERY OF THE EMBRYO FROM KCl POISONING WITHOUT THE AID OF ELECTROLYTES.

Eggs with normal heart beat were put for several hours (usually three and a half) into a  $M/2$  KCl solution to cause their hearts to stop beating.<sup>4</sup> They were then put into distilled water or different salt solutions to find out in which solution they recover most quickly. The most unexpected result obtained was that if the embryos had been sufficiently poisoned they would not recover at all or only in exceptional cases when put into distilled water or into a solution of saccharose, while they recovered rapidly when put into different salt solutions.

We will first discuss the fact that embryos whose hearts had stopped beating under the influence of a sufficient dose of KCl did not begin to beat when put into distilled water<sup>5</sup> or into a solution of saccharose, and that such eggs which had not recovered in distilled water after a number of days recovered in less than a day when put into a salt solution.

A number of eggs with embryos whose heart beat had developed were put into a  $M/2$  KCl solution for three and a half hours. Sixty eggs whose hearts had stopped beating were distributed in two dishes, one containing sea water, the other distilled water. Table III gives the rate at which the eggs recovered.

TABLE III.

After	Number of hearts which began to beat in	
	Sea water	H <sub>2</sub> O
<i>hrs.</i>		
1	0	0
2	2	0
3	7	0
4	11	0
5½	20	2
8½	27	2

<sup>4</sup> In a  $M/2$  KCl solution the hearts stop beating in less than 3½ hours, but the results are clearer if the eggs contain an excess of KCl.

<sup>5</sup> The reader will remember that distilled water is harmless for these eggs, and that the embryos of *Fundulus*, while marine organisms, will develop, hatch, and live in distilled water.

The next morning all thirty eggs had recovered in sea water and only three eggs in distilled water. The eggs which had not recovered in distilled water were then put into sea water, where they recovered at almost but not quite the same rate as those originally put into sea water (Table III), namely two after 2 hours, five after 4 hours, nine after  $5\frac{1}{2}$  hours, and twenty-two after 9 hours, had recovered and the next morning all had recovered, showing beating hearts.

A saccharose solution no more permits the recovery of the heart after KCl poisoning than does  $H_2O$ . Eggs that had been treated for three and a half hours with a  $M/2$  KCl solution and whose hearts had stopped beating were put into  $H_2O$ ,  $M/2$ ,  $M/8$ , and  $M/32$  saccharose, and into sea water, and the number of beating hearts was ascertained (Table IV). Twenty embryos were put into each solution.

TABLE IV.\*

After	Number of hearts which began to beat after standstill in KCl, in				
	Sea water	$H_2O$	$M/2$ saccharose	$M/8$ saccharose	$M/32$ saccharose
<i>hrs.</i>					
$1\frac{1}{2}$	1	1	0	0	0
3	7	2	0	0	0
$4\frac{1}{2}$	13	2	0	0	0
$7\frac{1}{2}$	18	2	1	0	0
$21\frac{1}{2}$	20	2	2	0	0
$47\frac{1}{2}$	20	3	2	1	0
96	20	2	0	1	0

\* Attention should be called to the fact that after 96 hours only two hearts were beating in  $H_2O$ , while three had been beating after  $47\frac{1}{2}$  hours. This observation is not uncommon in the recovery experiments and is due to the fact that a heart may beat for a little and then stop again without recovering permanently.

There was therefore in four days practically no recovery of the eggs which had been in  $H_2O$  or saccharose solutions, while the eggs put into sea water had almost all recovered in seven and a half hours.

The eggs that had not recovered in the  $H_2O$  or in the sugar solutions were not dead. In order to test this, those that had not recovered in  $H_2O$  and in  $M/32$  saccharose in four days were put into  $M/8$  solutions of NaCl,  $Na_2SO_4$ , NaI, and NaCNS. In six hours all or the majority of the eggs recovered.

The question arose, how long can the eggs poisoned with KCl live in distilled water without recovering and without losing the power of recovering when put into a salt solution?

Twenty-one eggs were put for five and a half hours into a  $M/2$  KCl solution and then transferred to  $H_2O$ , where they remained for seven days. During all this time only three hearts began to beat again. Nine of the remaining eighteen eggs were then put into  $M/8 Na_2SO_4$ ; after eighteen hours the hearts were beating in every one of the nine eggs. The other nine eggs remained for five days longer in distilled water during which time none of them began to beat again. They were then put into normal sea water and in six hours the hearts were beating in six of these eggs, but the others did not recover even after a longer exposure. This experiment, therefore, shows that some of the eggs which had not recuperated from KCl poisoning in twelve days were still able to recover in a short time when put back into sea water. The circulation was not established in these extreme cases, which indicates that the long cessation of the heart beat is not entirely harmless if the temperature is high, as was the case in these experiments.

These and many similar experiments show that the recovery of the *Fundulus* egg poisoned with KCl is only possible in a salt solution and not in distilled water nor in a solution of saccharose (and probably other non-electrolytes).

Only when the KCl has acted but a short time can the hearts recover in  $H_2O$  or a saccharose solution.

#### IV. THE RELATIVE EFFICIENCY OF DIFFERENT SALTS FOR THE RECOVERY OF EGGS POISONED WITH KCl.

We have seen that eggs poisoned with KCl will recover quickly when put into a salt solution but will not recover in  $H_2O$  or a saccharose solution. When put into a salt solution eggs poisoned with KCl will recover the more quickly the higher (within certain limits) the concentration of the salt of the surrounding solution. One experiment each of recovery in  $Na_2SO_4$  and NaCl of different concentrations may serve as examples. Both sets of experiments were made simultaneously. A large number of eggs of the same

age were put for three and a half hours into a  $M/2$  KCl solution and those whose hearts had stopped beating were selected for the experiment. Twenty eggs were put into each of the following solutions of  $Na_2SO_4$  and NaCl. As a control the recovery of such eggs in  $H_2O$  and in sea water was also noted (Tables V and VI).

TABLE V.

After	Number of hearts which began to beat in					
	$M/4$	$M/8$	$M/16$	$M/32$	$M/64$	$M/128 Na_2SO_4$
<i>hrs.</i>						
1	2	0	1	0	0	0
2	6	3	5	0	2	0
3	13	11	7	2	2	0
5	20	19	19	13	4	3
$7\frac{1}{2}$	20	20	20	18	11	8
19	20	20	20	20	19	17

TABLE VI.

After	Number of hearts which began to beat in						
	$H_2O$	Sea water	$M/2$	$M/8$	$M/16$	$M/32$	$M/128 NaCl$
<i>hrs.</i>							
1	0	1	1	0	0	0	0
2	0	3	4	3	0	0	0
3	0	6	10	4	1	0	0
5	0	10	20	17	7	2	0
$7\frac{1}{2}$	0	17	20	20	17	6	0
19	0	20	20	20	19	16	0

Several facts are worthy of notice. First, the tables show as stated that the hearts begin to recover the more quickly from the previous poisoning in KCl the more concentrated the solution. Second, a comparison of identical concentrations of NaCl and  $Na_2SO_4$  shows that the eggs recover more quickly in  $Na_2SO_4$  than in NaCl, and that the efficiency of  $Na_2SO_4$  is *more* than twice as great as that of NaCl for equal concentrations, since a  $M/64$  solution of  $Na_2SO_4$  corresponds in efficiency to a  $M/16$  solution of NaCl and a  $M/32$  NaCl solution to a  $M/128$  solution of  $Na_2SO_4$ . The third fact worth noticing is that sea water is less efficient than NaCl of the same osmotic pressure. We were also surprised to find (in experiments which we shall not record here) that the addition of  $CaCl_2$  to NaCl does not increase the inhibiting influence of the latter salt upon the diffusion of KCl into or from the egg.

We now wish to show that the effect of the salts upon the rate of recovery of the egg is influenced very strongly by the anion of the salt or salts added.

Eggs were put into a  $M/2$  KCl solution for three hours, and those in which the hearts had stopped beating were selected as usual for the experiment. Twenty eggs were put into the solution of the following different salts, all in a concentration of  $M/32$ , and a control of  $M/32$  sea water.

TABLE VII.

*Influence of Anion upon Number of Hearts Recovering from KCl.*

After	Sea water	NaCl	NaBr	NaI	NaNO <sub>3</sub>	NaCNS	Na acetate	Na <sub>3</sub> tartrate	Na <sub>2</sub> SO <sub>4</sub>	Na <sub>3</sub> citrate
<i>hrs.</i>										
2 $\frac{1}{2}$	0	2	3	4	2	0	2	6	5	10
4 $\frac{1}{2}$	2	4	6	8	5	4	6	12	13	14
5 $\frac{1}{2}$	5	9	8	10	11	7	12	14	19	11
9 $\frac{1}{2}$	11	14	18	17	16	20	19	19	20	Dead.

The most important results are those obtained after two and three-quarters and four and one-quarter hours. They show that the relative efficiency of the anions follows the order from the most efficient to the least efficient:

Citrate > sulphate > tartrate > acetate, iodide > Br > Cl, NO<sub>3</sub>.

The position of NaCNS is doubtful; it acts slowly, but all hearts finally recover. The sea water is less efficient even than NaCl, indicating an inhibiting effect of some substance contained in the sea water (Mg or NaOH?).

The case of Na<sub>3</sub> citrate requires a short discussion. It is obvious that at first the number of eggs which recover in Na<sub>3</sub> citrate is greater than in any of the other salts, but the superiority is only evident at first. This strange result finds its explanation in the fact that while the citrate is the most efficient ion in causing the recovery it is at the same time very toxic and after about four hours gradually kills the embryos. If we select a lower concentration in which the toxicity of the citrate is naturally less the greater relative efficiency of the citrate for recovery comes out very clearly.

In the following experiment (Table VIII) the number of eggs which recovered in very weak solutions of NaCl, Na<sub>2</sub>SO<sub>4</sub>, and Na<sub>3</sub> citrate was ascertained. The eggs had been kept for three hours in 3/8 M KCl and those whose hearts had stopped beating were selected for the experiment. Controls were made in H<sub>2</sub>O and normal (M/2) sea water. Only ten eggs were put into each solution.

TABLE VIII.

After	Number of eggs previously poisoned by KCl which recovered in										
	H <sub>2</sub> O	M/2 sea water	NaCl			Na <sub>2</sub> SO <sub>4</sub>			Na <sub>3</sub> citrate		
			M/64	M/128	M/256	M/64	M/128	M/256	M/64	M/128	M/256
hrs. 2	1	8	2	1	1	5	3	4	8	7	6

The experiment shows that M/256 Na<sub>3</sub> citrate in spite of its toxicity is indeed more efficient than M/64 Na<sub>2</sub>SO<sub>4</sub> and considerably more efficient than M/64 NaCl in accelerating the recovery of the eggs from KCl poisoning. The relative efficiency of the three salts increases therefore with the valency of the anion.

Experiments were made with phosphates to further test the idea concerning the influence of the valency of the anion. Na<sub>3</sub>PO<sub>4</sub>, Na<sub>2</sub>HPO<sub>4</sub>, and NaH<sub>2</sub>PO<sub>4</sub> were used. In the alkaline (Na<sub>3</sub>PO<sub>4</sub>) solution the eggs were killed too rapidly to give any result. The di- and monosodium phosphates were extremely efficient; the monosodium phosphate on account of its acidity could only be used in concentrations below M/32. The eggs were put for three and a half hours into M/2 KCl and those whose hearts had stopped beating were used for the experiment. Controls were made with H<sub>2</sub>O, sea water, and NaCl solution of various concentrations. Ten eggs were put into each solution.

It appears that in M/256 Na<sub>2</sub>HPO<sub>4</sub> the recovery is as rapid as in M/8 NaCl. NaH<sub>2</sub>PO<sub>4</sub> is apparently just as efficient as Na<sub>2</sub>HPO<sub>4</sub> except that the acid of the solution kills the eggs before the recovery is complete. The effects in the lower concentrations of NaH<sub>2</sub>PO<sub>4</sub> (M/128 and M/256) and after a short exposure (four to eight hours) are fully as good as those in Na<sub>2</sub>HPO<sub>4</sub>.

TABLE IX.  
*Recovery from KCl in NaCl and Phosphates.*

Nature of solution	Number of eggs previously poisoned by KCl which recovered after								
	1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	8 hrs.	20 hrs.	44 hrs.	7 days
H <sub>2</sub> O. ....		0	0	0	0	0	2	5	3
Sea water. ....	0	0	2	2	2	5	10		10
M/2 NaCl. ....	0	0	0	1	1	6	10		10
M/4 NaCl. ....		0	0	1	2	5	9	10	10
M/8 NaCl. ....			0	1	1	4	9	10	10
M/64 NaCl. ....				0	0	0	5	5	7
M/128 NaCl. ....				0		1	3	4	7
M/256 NaCl. ....						0	1	1	2
M/512 NaCl. ....							2	5	5
M/32 Na <sub>2</sub> HPO <sub>4</sub> . ....	1	5	7	7	9	10	10		
M/64 Na <sub>2</sub> HPO <sub>4</sub> . ....	0	1	2	3	4	7	10		
M/128 Na <sub>2</sub> HPO <sub>4</sub> . ....		1	1	2	4	6	9	10	
M/256 Na <sub>2</sub> HPO <sub>4</sub> . ....		0	0	1	1	2	9	9	
M/512 Na <sub>2</sub> HPO <sub>4</sub> . ....		0	0	0	0	0	2	6	
M/32 NaH <sub>2</sub> PO <sub>4</sub> . ....	0	0	1	2	2	1	0		
M/64 NaH <sub>2</sub> PO <sub>4</sub> . ....	0	3	5	5	7	3	0		
M/128 NaH <sub>2</sub> PO <sub>4</sub> . ....		0	1	2	5	6	2	1	
M/256 NaH <sub>2</sub> PO <sub>4</sub> . ....		0	0	1	3	3	8	8	
M/512 NaH <sub>2</sub> PO <sub>4</sub> . ....				0	0	2	2	9	

If we try to express the effect of the increasing valency of the anion in figures our results show that approximately

$$\begin{array}{ccccc} & \text{Na}_2\text{SO}_4 & & \text{Na}_3 \text{ citrate} & \\ \text{M/16 NaCl} = \text{M/64} & \text{or} & = \text{M/256} & \text{or} & \\ & \text{Na}_2 \text{ tartrate} & & \text{Na}_2\text{HPO}_4. & \end{array}$$

This approximates Hardy's rule that the ion effect should be an exponential function of the valency.

We feel justified in stating that the accelerating effect of salts upon the recovery of hearts previously poisoned with KCl is an anion effect inasmuch as it increases with the valency of the anion apparently in agreement with Hardy's rule, and inasmuch as the acetate is much more efficient than the chloride.

#### V. THE INFLUENCE OF THE CATION.

In the earlier experiments on the antagonization of KCl by NaCl and Na<sub>2</sub>SO<sub>4</sub> in the adult fish, Loeb and Wasteneys were inclined to



ascribe this effect to a competition between the Na and K for a common colloidal anion. This idea was based on the fact that  $\text{Na}_2\text{-SO}_4$  seemed to be about twice as efficient as NaCl; while in the case of an anion effect the efficiency of  $\text{Na}_2\text{SO}_4$  should have been greater than twice that of NaCl (according to Hardy's rule on the influence of valency upon the precipitating effect). The experiments on the adult fish were limited by the fact that the majority of Na salts (*e.g.*,  $\text{Na}_2$  tartrate,  $\text{Na}_3$  citrate, etc.) are so toxic that they could not be tested for their antagonistic effect upon KCl.

In the new experiments on the recovery of eggs poisoned with KCl these difficulties did not exist and Hardy's rule was confirmed.

That the greater efficiency of  $\text{Na}_2\text{SO}_4$  is due to the anion and not to the fact that this salt contains twice the amount of Na (though not twice the amount of Na ions) as NaCl, could be proved also by comparing the effect of  $\text{MgCl}_2$  and  $\text{MgSO}_4$  upon the recovery of eggs previously poisoned with KCl. Such an experiment is reported in Table X. Eggs were put into  $\text{m}/4$  KCl for twenty-four hours and those whose hearts had stopped beating were selected for a recovery experiment. Ten eggs were put into each of the following solutions,  $\text{m}/8$   $\text{MgCl}_2$ ,  $\text{m}/8$   $\text{MgSO}_4$ ,  $\text{m}/8$  NaCl,  $\text{m}/8$   $\text{Na}_2\text{SO}_4$ , and  $\text{H}_2\text{O}$ . The number of beating hearts in each solution was ascertained at various intervals.

TABLE X.

After	Number of beating hearts of eggs previously poisoned with KCl in				
	$\text{H}_2\text{O}$	$\text{m}/8 \text{ MgCl}_2$	$\text{m}/8 \text{ MgSO}_4$	$\text{m}/8 \text{ NaCl}$	$\text{m}/8 \text{ Na}_2\text{SO}_4$
<i>hrs.</i>					
2	0	1	2	3	5
5	0	2	6	5	8
11	1	3	9	7	10

The experiments show that  $\text{m}/8$   $\text{MgSO}_4$  is more than twice as efficient as  $\text{m}/8$   $\text{MgCl}_2$  although the concentration of the cation is the same in both solutions. This leaves no doubt that the difference in efficiency must be ascribed to the anion. At the same time it is obvious that  $\text{m}/8$  NaCl is more efficient than  $\text{m}/8$   $\text{MgCl}_2$  although the latter solution contains twice as much Cl as the former. This suggests the possibility that Mg may inhibit the recovery of the eggs from potassium poisoning, while the anions favor the recovery.

This idea led to an investigation of the effect of different cations upon the recovery of eggs previously poisoned by KCl. Eggs were put for twenty-five hours into  $M/4$  KCl and those whose hearts had stopped beating were selected for the experiment. They were then distributed into  $M/8$  LiCl, NaCl, RbCl, CsCl,  $NH_4Cl$ ,  $MgCl_2$ ,  $CaCl_2$ ,  $SrCl_2$ , and  $BaCl_2$  solutions and the number of those recovered (*i.e.*, whose hearts were beating) was ascertained. Ten eggs were put into each solution.

TABLE XI.

After	Number of hearts previously poisoned with KCl beating in									
	$H_2O$	$M/8$ LiCl	$M/8$ NaCl	$M/8$ RbCl	$M/8$ CsCl	$M/8$ $NH_4Cl$	$M/8$ $MgCl_2$	$M/8$ $CaCl_2$	$M/8$ $SrCl_2$	$M/8$ $BaCl_2$
<i>hrs.</i>										
2	0	7	5	0	1	4	2	4	1	0
5	1	7	8	1	1	5	2	7	2	2
10	1	10	10	2	1	6	3	6	2	3
48	0	10	10	1	0	6	5	6	4	1

Those eggs which had not yet recovered were then put into sea water to find out whether the salt solution had killed them or whether it had only prevented their recovery. The latter was the case with those in  $H_2O$ ,  $MgCl_2$ ,  $SrCl_2$ , RbCl, and  $NH_4Cl$ , which recovered very rapidly in sea water. In  $CaCl_2$  four had been killed by the  $CaCl_2$  and the same was true with some in  $BaCl_2$ . Those in CsCl recovered only very slowly, which may indicate a superposition of a Cs effect over that of K. Making allowance for such complications, the results are intelligible on the assumption that the anions of the solution (in this experiment the Cl ions) are responsible for the recovery of the eggs from KCl; and that the cations may have only a retarding effect. The latter is a minimum in the case of Li, is but slightly greater in the case of Na, and rises rapidly in the case of  $NH_4$ , Rb, and Cs. As far as the alkali earth metals are concerned, it is great in Mg, less in Ca, and is greater again in Sr and Ba. The writers make this statement about a possible retarding effect of the cations not without reluctance. We shall see in the next section that the hydrogen ion, though a cation, favors the recovery, while  $HO$ , though an anion, does not favor the recovery.

# VI. THE RÔLE OF ACIDS AND BASES IN THE RECOVERY OF ANIMALS POISONED WITH KCl.

It seemed of importance to ascertain the influence of acids and bases upon the recovery of embryos poisoned with KCl. The investigation of this problem is restricted by the high toxicity of both acids and bases, which when they diffuse through the membrane of the egg soon kill the embryo. It is, therefore, necessary to work with low concentrations of these substances and only consider the effect during the first few hours before the acid or alkali has had time to kill the embryo. Under such conditions it was found that if a trace of acid is added to distilled water the embryos may recover from potassium poisoning while otherwise they will not.

TABLE XII.

Nature of solution	Number of eggs previously poisoned in KCl recovered after					
	1 hr.	2 hrs.	3 hrs.	5 hrs.	10 hrs.	22 hrs.
H <sub>2</sub> O.....	0	0	0	1	2	2
Sea water.....	0	1	3	7	8	10
50 cc. H <sub>2</sub> O + 0.1 cc. N <sub>10</sub> acetic acid.....	0	2	3	4	6	3 (7 killed by acid).
50 cc. H <sub>2</sub> O + 0.2 cc. N <sub>10</sub> acetic acid.....	0	2	4	3	1 (5 killed by acid)	All killed by acid.
50 cc. H <sub>2</sub> O + 0.3 cc. N <sub>10</sub> acetic acid.....	1	1	2	3	1 (9 killed by acid)	All killed by acid.
50 cc. H <sub>2</sub> O + 0.4 cc. N <sub>10</sub> acetic acid.....	2	3	3	3 (3 killed)		

In one experiment eggs were put into a M/4 KCl solution for thirteen hours. Those whose hearts had stopped beating were distributed into the following solutions. Each solution contained ten eggs. (Table XII.)

It is obvious that the addition of 0.1 cc. N<sub>10</sub> acetic acid to 50 cc. of distilled water accelerates the recovery of the eggs almost as much as if they had been put into normal sea water. It is, moreover, obvious that the embryos are very soon killed by the acid itself as is indicated by the coagulation of such embryos. The acid becomes more efficient for the recovery of the eggs poisoned with KCl the

higher the concentration of the acid; but at the same time the eggs are killed more rapidly by the acid.

Since the point is of importance another experiment may be quoted. Eggs had been put into  $H_2O$  for twenty-four hours and only a few recovered. The others were distributed into various solutions and the number of recoveries is stated in Table XIII. Each solution contained ten eggs.

The accelerating influence of a trace of acid upon the recovery from KCl poisoning is unmistakable. Slight effects were also obtained with very weak HCl, and citric and tartaric acids.

All attempts to obtain similar effects with bases ( $NaOH$ ,  $NH_4OH$ ,  $Na_2CO_3$ ,  $Na_3PO_4$ ) were in vain.

It is also possible to slightly retard the poisoning of the embryos through the addition of a trace of acid to the KCl solution. These experiments are, however, not so striking, possibly on account of the disproportion between the concentration of KCl and acid.

TABLE XIII.

Nature of solution	Number of eggs which recovered from previous poisoning with KCl after					
	1 hr.	2 hrs.	3 hrs.	4 hrs.	6 hrs.	18 hrs.
$H_2O$ (21 eggs).....	0	0	2	2	2	3
m/8 sea water.....	0	1	4	5	7	10
50 cc. $H_2O$ + 0.2 cc. $\frac{N}{10}$ acetic acid	1	3	5	5	7	0 (all killed by acid).
50 cc. $H_2O$ + 0.4 cc. $\frac{N}{10}$ acetic acid	0	3	4	Killed by acid.		

#### VII. ON THE IMMUNIZATION OF THE EGGS AGAINST KCl BY DISTILLED WATER.

We now come to the description of a curious group of facts which we found in the course of these experiments. We had noticed that eggs treated for twenty-four hours with a very dilute solution of KCl, which did not stop their heart beat, were much more resistant to a KCl solution of higher concentration than eggs taken directly from normal sea water and put into a KCl solution of the same concentration. This observation (which might have suggested to a layman the possibility of an "adaptation" to the poison) induced us to try the effects of a previous treatment of the eggs with dis-

tilled water before they were put into  $M/2$  KCl. It was found that embryos which had been kept over night in  $H_2O$  were more resistant to KCl than eggs which had been put directly from sea water into a KCl solution of the same concentration. Thus in one case seventy-three eggs were put directly from sea water into a  $M/2$  KCl solution and the same was done with ninety-six eggs of the same lot which had been kept for about twelve hours in distilled water. After three hours all the hearts of the first lot stopped beating, while only twenty-two of the ninety-six eggs previously treated with  $H_2O$  had stopped beating. Finally the latter eggs also succumbed to the influence of KCl, but it required a considerably longer time. The  $H_2O$  had brought about a change in the egg which retarded the poisonous action of KCl upon the embryo. It may be stated again that the embryo of *Fundulus* develops as normally in  $H_2O$  as in sea water.

Systematic experiments were then made in which the eggs were put for different lengths of time into  $H_2O$  before they were put into the  $M/2$  KCl solution to find out how the  $H_2O$  would delay the action of KCl upon the eggs. The eggs were all of the same age. Twenty eggs were put directly from sea water into  $M/2$  KCl and the number of beating hearts was ascertained each hour. The next twenty eggs were put for one-quarter of an hour into  $H_2O$  before being put into the  $M/2$  KCl solution, the next lot of eggs were kept for one-half hour in  $H_2O$  before being put into the  $M/2$  KCl solution, and so on. Table XIV gives the result of such an experiment.

TABLE XIV.

*Influence of Previous Treatment of Eggs with  $H_2O$  upon the Rate of Poisoning by KCl.*

Time during which eggs had been in $H_2O$ before being put into $M/2$ KCl	NUMBER OF HEARTS BEATING IN $M/2$ KCl AFTER			
	1 hr.	2 hrs.	4 hrs.	24 hrs.
Not in $H_2O$ .....	4	3	1	0
15 min. ....	9	2	2	0
30 min. ....	12	4	3	0
1 hr. ....	15	5	3	0
2 hrs. ....	15	10	7	0
4 hrs. ....	16	10		0
6 hrs. ....	20			
22 hrs. ....	20	18	13	2

The result is most striking. When eggs are put directly from sea water into  $M/2$  KCl in one hour 80 per cent have no more heart beat; when the eggs are put for only fifteen minutes into  $H_2O$  before being put into the  $M/2$  KCl solution only 55 per cent of the hearts stop beating in one hour. When they are put for six hours into  $H_2O$  before being put into  $M/2$  KCl no hearts stop beating in one hour. When the eggs are put for a day into distilled water before being put into  $M/2$  KCl they show a still higher degree of resistance to KCl.

This resistance to KCl is reversed but slowly when the eggs are put back into sea water after the  $H_2O$  treatment. It was found that in the eggs of one set all the hearts stopped beating when the eggs had been in  $M/2$  KCl for two hours. Eggs of the same set that had previously been put for forty-seven hours into distilled water had all (with the exception of one) beating hearts after they had been exposed to the  $M/2$  KCl solution for two hours. A third group of the same eggs was put into  $H_2O$  for twenty-four hours, then into sea water for eighteen, and was then submitted to  $M/2$  KCl (simultaneously with the two other sets). After two hours, six hearts of this lot were still beating.

#### VIII. THE INFLUENCE OF THE CONCENTRATION OF ELECTROLYTES UPON THE SUBSEQUENT EFFECT OF KCl.

It may be well to discuss briefly a theoretical point before we go further. When eggs were poisoned with KCl so that the hearts had stopped beating they did not as a rule recover when put into distilled water. Since the recovery cannot take place unless the KCl diffuses out of the egg we will assume that the eggs do not recover in distilled water from KCl poisoning because the KCl cannot diffuse from the egg into the distilled water. This assumption will also explain why eggs which have been put for some time into  $H_2O$  will be poisoned much more slowly when put afterwards into a  $M/2$  KCl solution. If we assume that the immersion of the eggs in the distilled water causes the formation of a layer of distilled water in the network of fibrils forming the egg membrane we can understand that such a layer of  $H_2O$  forms as efficient a barrier against the

diffusion of KCl through the membrane into the egg, as does the  $H_2O$  for the diffusion of KCl from the egg previously poisoned with KCl when such an egg is put into distilled water.

Considerations of this kind led us to expect that when we put eggs for some time into different concentrations of a salt solution previously to putting them into  $M/2$  KCl the eggs should be the more resistant to the KCl the lower the concentration of the salt in which they had previously been kept.

Twenty eggs were placed for eleven hours into each of the following solutions:  $M/2$  and  $M/8$  sea water, and  $H_2O$ . From here they were transferred into  $M/2$  KCl and the rate at which they were poisoned was ascertained. Each solution contained twenty eggs. Table XV gives the number of embryos whose hearts were still beating.

TABLE XV.

Eggs 11 hrs. in	Number of hearts beating in $M/2$ KCl after			
	1 hr.	5 hrs.	13 hrs.	24 hrs.
$H_2O$ .....	20	19	16	13
$M/2$ sea water .....	3	2	0	0
$M/8$ sea water .....	20	17	8	0

While the embryos transferred into  $M/2$  KCl from  $M/2$  sea water had practically all ceased to have a heart beat after one hour (only three of twenty had heart beats) the hearts of those from  $M/8$  sea

TABLE XVI.

Eggs previously kept for $12\frac{1}{2}$ hrs. in	Number of hearts beating in $M/2$ KCl after				
	1 hr.	2 hrs.	$3\frac{1}{2}$ hrs.	6 hrs.	30 hrs.
$M/16$ NaCl .....	18	10	5	1	0
$M/32$ NaCl .....	19	8	2	1	0
$M/64$ NaCl .....	19	12	6	2	0
$H_2O$ .....	19	17	8	3	0
$M/2$ sea water .....	7	1	1	0	

water and from  $H_2O$  were at that time all still beating. The immunity induced by  $H_2O$  was, naturally, of greater duration than that given by  $M/8$  sea water.

An experiment with various low concentrations of NaCl con-

firms this result and shows that below  $m/8$  solutions a further lowering of the concentration of NaCl has comparatively little influence. Eggs were kept for twelve and one-half hours in  $m/16$ ,  $m/32$ ,  $m/64$  NaCl, and  $H_2O$  and then transferred into  $m/2$  KCl. Twenty eggs were used in each solution. Table XVI gives the result.

While the eggs taken from sea water succumbed to the KCl in less than two hours those from  $H_2O$  had practically all beating hearts at that time (seventeen out of twenty). Those from  $m/16$ ,  $m/32$ , and  $m/64$  NaCl were about midway between those from sea water and from  $H_2O$ .

Eggs that had been kept in weak KCl solutions without succumbing to the KCl also showed the effect of the dilution, *i.e.*, a greater immunity to  $m/2$  KCl.

#### IX. THE RELATIVE TOXICITY OF DIFFERENT POTASSIUM SALTS.

The experiments thus far mentioned indicate that anions retard the diffusion of potassium into the egg, and accelerate such a diffusion out of the egg, and that this effect increases with their valency of the anion and is greater for acetate than for Cl. We have compared the relative toxicity of equimolecular concentrations of KCl, K acetate, and  $K_2SO_4$ . We should expect that in regard to toxicity the order should be  $KCl > K \text{ acetate} > K_2SO_4$ , provided that the anions have an inhibiting effect upon the diffusion into the egg. For such experiments concentrations of  $m/8$  or above must be used since we shall see later that concentrations of KCl below  $m/8$  are so little poisonous that they cannot be used for obtaining an answer to our question. We give two series with  $m/8$  and  $m/2$  solutions of KCl, K acetate, and  $K_2SO_4$ . Twenty eggs of the same set were put into each solution and the number of embryos with beating hearts was determined after certain intervals. Table XVII gives the result.

If we compare the effect of  $m/8$  K acetate with that of  $m/8$  KCl we notice that K acetate is less toxic than KCl; and the same is true for  $K_2SO_4$ , although in the latter solution the concentration of K is twice as great as in KCl.

The same result appears in the  $m/2$  solutions though not quite so



strikingly. These experiments therefore conform with the facts put into evidence in the previous chapters of this paper showing that the anions inhibit the diffusion of K into the egg.

TABLE XVII.\*

In	Number of embryos with hearts beating after							
	$\frac{1}{2}$ hr.	1 hr.	2 hrs.	hrs.	5 hrs.	9 hrs.	25 hrs.	60 hrs.
M/8 KCl.....	15	11	8	8	8	7	7	1
M/8 K acetate.....	20	18	14	12	14	15	14	1
M/8 K <sub>2</sub> sulphate.....	18	14	12	12	12	13	12	8
M/2 KCl.....	14	8	2	0	0	0	0	0
M/2 K acetate.....	17	7	4	1	1	0		
M/2 K <sub>2</sub> sulphate.....	15	7	0	0	0			

\* It should be stated that the sensitiveness of eggs to KCl solutions of lower concentrations differs slightly for eggs of different age and possibly also of different females. Thus the eggs used in Tables I and II were more sensitive than those used in Table XVII.

We have mentioned that the lower concentrations of K salts are very little poisonous for the embryo. This fact is very remarkable and deserves attention. In Table XVIII are given the results of an experiment on the relative toxicity of different concentrations of KCl.

TABLE XVIII.

In	Number of embryos with beating hearts after									
	$\frac{1}{2}$ hr.	1 hr.	1½ hrs.	2 hrs.	2½ hrs.	48 hrs.	76 hrs.	105 hrs.	192 hrs.	288 hrs.
M/2 KCl.....	9	2	0	0	0	0	0	0	0	0
M/4 KCl.....	16	9	7	4	4	4	1	0		
M/8 KCl.....	19	15	11	14	16	16	13	12	6	0
M/16 KCl.....	20	15	15	16	16	15	14	12	3	0
M/32 KCl.....	20	20	20	20	20	19	19	19	5	0
M/64 KCl.....	20	19	19	19	20	20	20	18	5	0
M/128 KCl.....	20	20	20	20	20	20	20	20	6	1

Twenty eggs (twelve days old) were put into each of the following KCl solutions and the rate at which the hearts stopped beating was ascertained.<sup>6</sup>

<sup>6</sup> It is possible that the hearts of younger embryos are affected more quickly by lower concentrations than the hearts of older embryos.

The striking fact is that a  $M/32$  KCl solution or below is not toxic while a  $M/16$  solution is but slightly toxic. A  $M/16$  KCl solution has approximately three times as high a concentration of KCl as that in which this salt is contained in the sea water. Beginning with  $M/8$  the toxicity rises rapidly. The explanation is not simple. We might make the assumption that, beginning with very low concentrations, the inhibiting effect of the anion increases at first very rapidly with the increasing concentration of the anion, but after that increases more slowly with increasing concentration.

There is another explanation possible, based on the fact that if eggs are treated with  $H_2O$  or with a weak solution of some salt they become more resistant to KCl. It may be that in a weak solution of KCl the dilution effect makes itself felt, inasmuch as inside the meshes of the fibrils of the membrane of the egg a layer of  $H_2O$  or of very dilute KCl solution is formed which acts as a barrier to the further diffusion of KCl into the egg.

#### X. THEORETICAL REMARKS.

We have seen in this paper that when an embryo is poisoned by a potassium salt it cannot recover in  $H_2O$  or a saccharose solution; but that it will recover when put into the solution of an electrolyte; and that the latter's efficiency increases with the valency of the anion. As far as we can see there are two ways in which these facts might be explained: first, by assuming that the recovery is due to the outside electrolytes diffusing into the egg and acting directly on the embryo or heart, whereby the latter recovers from its standstill caused by potassium. The second possible explanation is based on the assumption that the recovery of the embryo whose heart has stopped beating depends on the diffusing of the KCl out of the egg into the surrounding solution. On the basis of this assumption we should be forced to conclude that the diffusion of KCl from the membrane of the egg into  $H_2O$  or a sugar solution is practically impossible and that the surrounding solution must contain a certain concentration of anions which may be the smaller the higher their valency in order to allow the potassium to diffuse out.

It can be shown that the latter assumption is more probable, since

$\text{Na}_3$  citrate,  $\text{Na}_2\text{HPO}_4$ , and  $\text{Na}_2$  tartrate, which favor the recovery of the poisoned egg of *Fundulus*, are so toxic for the fish after it has left the egg that they kill it very rapidly; and that they cannot be used for the antagonization of potassium effects on the adult fish, since they kill the latter in much smaller doses than those required for the recovery of the embryo. Hence the efficiency of these anions for the egg must be based on the fact that they do not diffuse into the egg. As a matter of fact, we had to mention, in the discussion of our experiments on citrates, that only the results of experiments with low concentrations of this salt and of short duration could be used for the egg, since after a little while the citrate entering into the egg killed the embryo. It is, therefore, not possible to assume that the great effect of the citrates upon the recovery of eggs previously poisoned with KCl is due to a direct action of these salts on the embryo.<sup>7</sup> A second argument in favor of the diffusion theory is the fact that if eggs are put for some time into  $\text{H}_2\text{O}$  before being put into the potassium solution, the latter will poison the egg much more slowly. This is only intelligible on the basis of the assumption that the diffusion of KCl is retarded by the previous treatment with  $\text{H}_2\text{O}$ , since  $\text{H}_2\text{O}$  is not antagonistic to the toxic effects of KCl.

We are then driven to the conclusion that the action of electrolytes upon the prevention and the reversal of potassium poisoning in the embryo of *Fundulus* is due to an influence of these electrolytes upon the rate of diffusion of potassium through the membrane.

On this assumption our results would lead us to the conclusion that *the anions of the surrounding solution retard the diffusion of the K ions into the membrane (and the egg) and accelerate the diffusion of the K ions out of the membrane (and the egg)*. If the egg is poisoned with KCl and if it is put into  $\text{H}_2\text{O}$  or a saccharose solution, the egg cannot recover on account of the lack of anions in

<sup>7</sup> In passing we might remark that the former experiments on antagonistic salt action in the embryo of *Fundulus* all indicated that the antagonistic action consisted in the prevention of the diffusion of the outside salt solution into the egg. Thus the *Fundulus* embryo, as long as it is inside the egg membrane, will keep alive and float in a solution of 50 cc. 3 M NaCl + 1 cc. 10/8 M  $\text{CaCl}_2$  for five days, while the newly hatched fish is killed almost instantly in such a solution. A 10/8 M solution of NaCl +  $\text{CaCl}_2$  is the upper limit in which the newly hatched fish can live. This topic has been sufficiently discussed in former papers to which the reader may be referred.

this solution. If normal eggs are put for some time into distilled water the latter enters into the meshes of the felt-like membrane. If such eggs are subsequently put into  $M/2$  KCl the layer of distilled water inside the membrane acts as a barrier through which the progress of the diffusion of the K ions into the eggs is retarded; since the more peripheral fibers of the membrane containing potassium can no more give it off to a layer of distilled water inside the membrane than they can to distilled water on the outside. Finally, when normal eggs are put into a KCl solution the retarding influence of the anions of the K solution is increased by the influence of the anions of the electrolyte added, although this action is partly balanced by the cations of the salt added.

It is also of importance to point out that while the eggs poisoned with KCl can easily recover in LiCl or NaCl (and in the former more quickly than in the latter) they recover not at all or only slowly in solutions of RbCl or CsCl. It is also possible that bivalent cations directly inhibit the recovery since in NaCl the eggs recover more quickly than in  $MgCl_2$  or  $CaCl_2$ .

In a former paper<sup>8</sup> one of us has already pointed out that the potassium behaves in these experiments very much like an invisible basic dye. When we stain the egg membrane with a basic dye, like neutral red, the membrane is readily decolorized when the stained eggs are afterwards put into salt solutions, while the stained eggs are not or are only very slowly decolorized when put into distilled water. If we try to stain eggs in a neutral red solution to which salt is added, we also notice a retardation of the staining. These facts suggest that the diffusion of KCl or of K through the membrane of the egg is a process which, in its initial stage at least, is analogous to the diffusion of a basic dye through the membrane. The common basis for both phenomena lies in the nature of the forces by which neutral red and K are held in the membrane. We may imagine that there exists in the membrane a colloidal anion to which the cation, like K or neutral red, is bound. These binding forces are counteracted by the anions of the surrounding solution.

The analogy between the behavior of potassium and neutral red shows itself also in the fact that when eggs stained with neutral red

<sup>8</sup> Loeb, *Proc. Nat. Acad. Sc.*, 1915, i, 473.

are put into an acid solution the stain is readily given off; and we have also seen that when the eggs are poisoned with potassium they give off the potassium readily in a faintly acid solution. This acid effect can be explained by assuming that the colloid of the membrane which binds the potassium or the neutral red cation is an amphoteric electrolyte, which forms a salt with the acid; this salt is comparatively strongly dissociated, the colloid becoming the cation. This colloidal cation is no longer able to bind the potassium or neutral red cation.

Another point requires discussion. It is much easier to demonstrate the accelerating influence of electrolytes upon the recovery of the heart (or the diffusion of KCl out of the egg) than the retarding influence of electrolytes upon the diffusion of KCl into the heart. The reason is probably this, that in the latter case the KCl must be present in rather high concentration in the outside solution while in the former case the concentration of KCl in the outside solution is almost zero, since it will contain only the traces of KCl which diffuse out of the egg.

It is also obvious that these experiments, in case they can be generalized, must lead us to a new conception of the mechanism of the diffusion of cations through membranes, inasmuch as the chemical or kindred forces which colloidal anions of the membrane exercise upon the diffusing cation seem to play a decisive rôle in the mechanism of diffusion. This has already been mentioned by one of us in a previous paper.

Donnan has developed the equations for the equilibrium of distribution of cations between diffusible and non-diffusible (colloidal) anions separated by an animal membrane. It is quite possible that his equations cover our results as far as cations are concerned. Besides the Donnan effect we have, however, a marked anion effect which is not covered by his theory.

#### XI. SUMMARY OF RESULTS.

1. It is shown in this paper that eggs of *Fundulus* poisoned with KCl are not able to recover when put into distilled water or a saccharose solution, while they will recover when put into a solution of a salt or when a trace of acid is added to the distilled water. Hearts

which have not been able to recuperate when kept for days in solutions of non-electrolytes will recover quickly when put into salt solutions. The indicator for the potassium poisoning is the standstill of the heart of the embryo and for the recovery the resumption of the heart beats.

2. It is shown that the relative efficiency of the salts for inducing the recovery of the heart beat increases, first (within certain limits), with the concentration of the salt in the solution, and second, with the valency of the anion of the salt, the valency effect apparently following Hardy's rule.

3. One of us has already pointed out in a previous publication that this action of the egg towards potassium is somewhat analogous to its behavior towards neutral red. When *Fundulus* eggs, stained with neutral red, are put into distilled water they cannot give off their stain; they give it off, however, when a trace of acid or some salt is added.

4. The behavior of both the basic dye and the potassium can be understood on the assumption that their diffusion presupposes their combination with a colloidal anion of the membrane. This combination is counteracted by the presence of an excess of anions, especially di- and trivalent ones, in the outside solution, and it is also counteracted by the presence of a trace of acid in the outside solution.

5. This action of the acid may be explained on the assumption that the colloid of the membrane which binds the potassium and the neutral red is an amphoteric electrolyte which through the addition of the acid is transformed into a salt, in the dissociation of which the colloid forms a cation which is no longer able to bind other cations.

6. It is shown that if eggs are previously treated with distilled water for some time the KCl requires a much longer time to bring about the poisoning than if the eggs are put into the KCl solution directly from sea water. This can be explained on the assumption that by the immersion of the egg in distilled water traces of it will get into the network of fibrils constituting the membrane and this layer of  $H_2O$  will act as a barrier blocking the further diffusion of the potassium through the membrane as effectively as did the distilled water surrounding the membrane in the experiment mentioned in 1.

## ON THE RÔLE OF ELECTROLYTES IN THE DIFFUSION OF ACID INTO THE EGG OF FUNDULUS.

By JACQUES LOEB.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, September 17, 1915.)

### I.

The experiments of Loeb and Cattell<sup>1</sup> on the diffusion of potassium into or from the egg of *Fundulus* have shown that this process is influenced by the anions in a way suggestive of the Hofmeister series and of Hardy's valency rule. It was of interest to find out whether other cations besides K showed a similar behavior. The writer tested the diffusion of acetic acid into the egg. It was found that this process is retarded by the anions in the same but still more pronounced way than was true for the diffusion of K; but that in addition the counteracting effect of the cation is equally marked and increases also with increasing valency.

The acetic acid was used in concentrations of M/500 (1 cc. M/10 acetic acid in 50 cc. solution). The criterion for the toxic action of the acid was the standstill of the heart of the embryo inside the egg. The eggs used were always over four days old. Soon after the standstill of the heart the embryo and yolk sac underwent coagulation which betrayed itself by the white opaqueness. It was found that the time in which the hearts stopped beating was much shorter when the acid was added to distilled water than when it was added to salt solutions. We will first show that the antagonistic effect of the salt solution increases, within certain limits, with its concentration. Table I may serve as an example. The acid was

<sup>1</sup> Loeb, J., *Proc. Nat. Acad. Sc.*, 1915, i, 473. Loeb, J., and Cattell, McK., *Jour. Biol. Chem.*, 1915, xxiii, 41.

M/500 acetic acid, the salt was NaCl. Ten eggs were put into each solution.

TABLE I.

After	Number of embryos with heart beat in 1 cc. M/10 acetic acid in 50 cc.					
	H <sub>2</sub> O	M/64	M/32	M/16	M/8	M/4 NaCl
hrs.						
4	3	0	0	4	10	10
5½	0	0	0	1	8	10
6½	0	0	0	0	8	10
9	0	0	0	0	3	8
20	0	0	0	0	0	0

It is obvious that the M/4 NaCl retards the velocity with which the embryos are killed in the acid more efficiently than do the weaker concentrations of NaCl. Very weak salt solutions like M/64 or M/32 may possibly be more harmful than distilled water. An analogue to such a possibility was found in the earlier experiments of Loeb and Wasteneys on the counteraction of the poisonous effects of KCl by NaCl.<sup>2</sup> We will show in Table II that the protective action of salts is a distinct function of the nature and valency of the anion. Each solution contained ten eggs.

TABLE II.

After	Number of embryos with heart beat in 1 cc. M/10 acetic acid in 50 cc.								
	H <sub>2</sub> O	M/8 NaCl	M/8 NaBr	M/8 NaI	M/8 NaNO <sub>3</sub>	M/8 Na acetate	M/8 NaSCN	M/8 Na <sub>2</sub> SO <sub>4</sub>	M/8 Na <sub>2</sub> tartrate
hrs.									
5½	0	9	10	0	1	10	10	10	10
7½	0	0	1	0	0	10	10	10	10
10½	0	0	0	0	0	10	10	10	10
24	0	0	0	0	0	10	10	10	10
48						8	10	10	10
96						7	10	7	10

It would be difficult to find a more striking demonstration of the rôle of the anion in the counteraction of the toxic action of the acid, the organic anions and the bivalent ones being much more

<sup>2</sup> Loeb, J., and Wasteneys, H., *Biochem. Ztschr.*, 1911, xxxii, 155.



efficient than the inorganic univalent anions, Cl, Br, I, and  $\text{NO}_3$ . The order of efficiency from the weakest to the strongest antagonist is



The result is similar to the antagonization of potassium poisoning, except that SCN is much more active in this case than in the case of potassium.

The same order in the relative efficiency of the anion was also found in the case of other salts, *e.g.*,  $\text{NH}_4$  and Li, as shown in Table III.

TABLE III.

After	Number of beating hearts in 1 cc. m/10 acetic acid in 50 cc. "					
	H <sub>2</sub> O	m/8 NH <sub>4</sub> Cl	m/8 NH <sub>4</sub> NO <sub>3</sub>	m/8 NH <sub>4</sub> acetate	m/8 (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	
hrs.						
3	5	9	9	10	9	
8½	0	0	0	10	8	
24	0	0	0	10	5	
48	0	0	0	9	2	
	H <sub>2</sub> O	m/8 LiCl	m/8 LiBr	m/8 LiI	m/8 Li acetate	m/8 Li <sub>2</sub> SO <sub>4</sub>
6½	5	10	9	0	10	10
10½	0	5	2	0	10	10
24	0	0	0	0	8	10
48	0	0	0	0	8	2
96	0	0	0	0	7	1

Acetate and sulphate are again much more efficient antagonists of acid than Cl, Br, I, and  $\text{NO}_3$ . I is the most inefficient of all anions. m/8  $\text{MgSO}_4$  was also much more efficient than m/8  $\text{MgCl}_2$ .

While these results agree in general with those on the diffusion of K through the membrane of the egg, the following observations differ from those made in the case of K. We had seen that  $\text{MgCl}_2$  counteracted the action of potassium less than NaCl. In the case

of the prevention of the action of the acid it is just the reverse. The antagonistic action of the salts with bivalent cations is very much greater than that with univalent cations. This is indicated by Table IV. Ten eggs with embryos with beating hearts were put into each solution.

TABLE IV.

After	Number of embryos with beating hearts in 1 cc. M/10 acetic acid in 50 cc.									
	H <sub>2</sub> O	M/8 LiCl	M/8 NaCl	M/8 KCl	M/8 RbCl	M/8 CsCl	M/8 MgCl <sub>2</sub>	M/8 CaCl <sub>2</sub>	M/8 SrCl <sub>2</sub>	M/8 BaCl <sub>2</sub>
hrs.										
4½	0	10	10	3	4	5	10	10	10	6
8½	0	1	2	3	2	2	8	10	10	5
24	0	0	0	0	0	0	3	10	10	0
72							1	10	10	0
96							1	10	10	0

CaCl<sub>2</sub>, SrCl<sub>2</sub>, and to some extent MgCl<sub>2</sub>, are much stronger antagonists to acid than were the chlorides of monovalent metals. The hearts stopped beating in KCl, RbCl, and CsCl more rapidly than in LiCl and NaCl, presumably because the action of K, Rb, and Cs on the heart beat was superposed on that of the acid. The difference in the efficiency of M/8 NaCl and M/8 CaCl<sub>2</sub> can not be attributed to the higher concentration of Cl ions in the CaCl<sub>2</sub> solution since we have seen that the difference in the action of M/8 and M/4 NaCl (Table I) is very much less than that between M/8 NaCl and M/8 CaCl<sub>2</sub>.

## II.

Loeb and Cattell have mentioned that the presence of acid retards the diffusion of potassium into the egg of *Fundulus*. We can show that the same is true for the diffusion of Ca into the egg. In a previous paper we have shown that a 3/16 M CaCl<sub>2</sub> solution kills the eggs of *Fundulus* in a couple of days or less. It can easily be shown that acid prevents or retards this effect. It is possible to use a comparatively high concentration of acid in this case, since the CaCl<sub>2</sub> renders the acid rather harmless. M/500 acetic acid and M/5,000 HCl acted best. Bases had no such action. It is not

advisable to give figures, since the writer intends to continue these experiments.

### III. THEORETICAL REMARKS.

It is obvious that in order to kill the embryo the acid must diffuse through the membrane of the egg and the question arises whether the salt in the outside solution inhibits or retards this diffusion; or whether it diffuses with the acid into the egg and prevents the injurious action of the acid upon the embryo inside the membrane.

Loeb and Wasteneys have shown that salts inhibit also the action of acid upon the adult fish, but that this action is practically restricted to chlorides.<sup>3</sup> Tartrates or rhodanates are too toxic to be useful for this purpose. This would indicate that in our recent experiments the antagonistic action of the salt must have consisted in retarding or preventing the diffusion of enough acid into the egg to injure the embryo, since we found SCN and tartrate very efficient in the prevention of acid poisoning of the embryo.

In their earlier experiments on the inhibition of the action of acid on the fish Loeb and Wasteneys have shown that in a pure acid solution the fish dies very rapidly, obviously through suffocation, the gills becoming unfit for respiration. This action is inhibited or retarded by salts. A titration of the acidity of the solution showed no noticeable difference in the absorption of the acid in the presence or absence of salts. This seems to contradict our conclusion that in the embryo the action of the salt consists in its influence on the diffusion of the acid through the membrane. But it is obvious that the two cases differ in this, that the action of the acid on the surface of the egg membrane does not injure the embryo directly, while the action on the surface is decisive in the case of the gills.

We must apparently discriminate between the action of two portions of the acid, one combining with a colloid of the membrane of the egg or cell and forming a salt, and the other portion diffusing into the egg and killing the embryo. In the case of the adult fish it is obviously the first portion acting on the surface of the gill leaves which kills the fish by suffocation. In the case of the egg

<sup>3</sup> Loeb and Wasteneys, *Biochem. Ztschr.*, 1911, xxxiii, 489; 1912, xxxix, 167.

it is the second portion which kills the embryo. When tartrates or SCN inhibit the action of acid on the embryo it is obviously the effect of the second portion which is inhibited. It is possible that the process of the diffusion of acid is the same as that discussed in the case of potassium and on this supposition the action of the anion is intelligible. It would be of importance to see whether the poisonous action of the acid on the embryo is also reversible, at least in its initial stage, and whether the reversibility resembles that of the reversibility of the potassium poisoning reported in the paper by Loeb and Cattell. It may be difficult to decide this question. We must also consider the possible influence of the water absorbed by the membrane on the rate of diffusion of acid into the egg. The writer had shown long ago that the muscle absorbs water under the influence of acid<sup>4</sup> and that this process is inhibited by salts.<sup>5</sup> It is possible that the diffusion of acid through the membrane is facilitated by the swelling of the membrane by acid, and that the antagonistic influence of the salt might be due to the antagonization of the swelling.

#### SUMMARY OF RESULTS.

1. It is shown that salts inhibit the toxic action of acids upon the embryo of *Fundulus*.

2. This inhibiting action of the salts is a function of the anion as well as the cation. Rhodanates, acetates, sulphates, and tartrates inhibit very strongly, chlorides, bromides, and nitrates much less, and iodides least of all. The bivalent cations Ca and Sr, and to a smaller degree Mg, also inhibit more strongly than the univalent cations.

3. Since tartrates and rhodanates are much too toxic to be of use in inhibiting the antagonistic action of acids upon the adult fish we must conclude that the antagonistic action of the anions in our experiments consisted in retarding the rate of diffusion of the acid through the membrane.

<sup>4</sup> Loeb, *Arch. f. d. ges. Physiol.*, 1898, lxi, 1; 1898, lxxi, 457.

<sup>5</sup> Loeb, *ibid.*, 1899, lxxv, 303.

## NOTE ON THE APPARENT CHANGE OF THE OSMOTIC PRESSURE OF CELL CONTENTS WITH THE OSMOTIC PRESSURE OF THE SURROUNDING SOLUTION.

By JACQUES LOEB AND HARDOLPH WASTENEYS.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, September 17, 1915.)

### I.

Loeb and Cattell<sup>1</sup> have shown that if the eggs of *Fundulus* are previously kept for some time in distilled water or in a very weak salt solution, they offer a greater resistance to the poisonous effects of a  $M/2$  solution of KCl than if they are transferred to the  $M/2$  KCl solution from sea water. This experiment is explained on the assumption that between the meshes of the fibers constituting the membrane the distilled water or weak salt solution collects and forms a barrier to the diffusion of the KCl through the membrane. The same authors have shown that if eggs are poisoned with KCl they do not recover, or recover only very slowly, when put into distilled water or sugar solutions, while they do recover when put into solutions of electrolytes. It seemed, therefore, advisable to ascertain whether when *Fundulus* eggs are put from sea water into distilled water or into weak concentrations of salt solutions some of the distilled water or weak salt solution will collect in the meshes of the membrane, replacing the sea water formerly occupying the same space. If this idea is correct, the freezing point of the eggs must change considerably with the concentration of the solution, in which the eggs are previously kept or washed. On the assumption that the membrane is practically impermeable to water and salt in physi-

<sup>1</sup> Loeb, J., and Cattell, McK., *Jour. Biol. Chem.*, 1915, xxiii, 41.

ologically balanced salt solutions and in distilled water (which was made probable in previous publications by Loeb), the osmotic pressure of the juice pressed out from eggs of *Fundulus* should be the mean of the following two solutions: (1) the contents of the egg, which are constant for the same set of eggs; and (2) the solution absorbed between the meshes of the outer fibrils of the membrane. Only the latter liquid would vary with the concentration of the outside medium.

## II.

Fertilized eggs of *Fundulus* were kept for a day or longer in distilled water,  $M/2$ , or gram molecular sea water. They were then rinsed several times in tap water and put for a few minutes in distilled water. They were then taken out, drained, rubbed gently between two sheets of filter paper to free them from the water adhering to the outside; then mixed with sand, put into canvas, and their juice was pressed out in a Buchner press. Enough eggs were taken to obtain a quantity of juice sufficient for the determination of the freezing point depression. Table I gives the result of some experiments.

TABLE I.  
*Fertilized Eggs.*

Eggs kept for 24 hrs. or more previously in	Then washed in	$\Delta$ of egg content.
Distilled water.....	Distilled water	0.42°
Distilled water.....	Distilled water	0.47°
$M/2$ sea water.....	Distilled water	0.49°
$M$ sea water.....	Distilled water	0.57°

This table shows the after effect of the solution in which the eggs had been kept previous to the washing, inasmuch as the eggs that had been kept in  $M$  sea water had a freezing point depression of 0.57°, while those that had been kept in  $M/2$  sea water had a freezing point depression of 0.49°, and those kept in distilled water had a freezing point depression of 0.42°. They may have been kept longer than one day in distilled water, although we have no record for this.

This table does not allow us to recognize the result of the washing. The influence of the washing is shown in the next set of ex-

periments. Eggs that had been kept in sea water were washed in sea water diluted with different quantities of distilled water. After this they were freed from the water adhering to the outside by rolling them gently between filter paper. The freezing point depression of the water in which the eggs were washed was measured and is given in the second column of Table II. It is obvious that the depression of the freezing point of the juice from the eggs increases with the depression of the freezing point of the wash water and is always higher than that of the eggs of Table I which had been washed with distilled water.

TABLE II.

Eggs previously kept in	Then washed in sea water with freezing point depression of	$\Delta$ of egg content.
Sea water.....	0.52°	0.66°
Sea water.....	0.55°	0.68°
Sea water.....	0.67°	0.72°
Sea water.....	0.68°	0.75°
Sea water.....	0.80°	0.77°
Sea water.....	1.04°	0.88°
Sea water.....	1.93°	1.27°

Two facts stand out clearly. First, that the osmotic pressure of the juice of the eggs grows with the concentration of the wash water, and, second, that while the osmotic pressure of the egg contents is above that of the wash water as long as the latter is  $< 0.68^\circ$ , it falls below it as soon as the  $\Delta$  of the wash water becomes  $0.80^\circ$  or more. On the reasoning given above, this would indicate that the real freezing point depression of the contents of the egg seems to lie between  $0.75^\circ$  and  $0.77^\circ$ .

In a third group of experiments the eggs were always washed in a mixture of sea water and  $H_2O$ , with a freezing point depression of  $0.67^\circ$ , but the eggs had been kept twenty-four hours previous to the experiment in solutions of different concentrations. This experiment was made to make sure that when the eggs are exposed to another solution for a longer time previous to the washing, the brief washing will not necessarily eliminate this solution completely from the meshes inside the membrane.

The influence of the concentration in which the eggs had been kept before washing showed itself, but was not regular. This latter

fact can be understood, since slight differences in the structure of

TABLE III.

Eggs previously kept in	Washed in diluted sea water of	$\Delta$ of egg content.
M/2 sea water .....	0.67°	0.71°
M/2 sea water .....	0.67°	0.81°
M sea water .....	0.67°	0.80°
M sea water .....	0.67°	0.99°
Distilled water .....	0.67°	0.64°
Distilled water .....	0.67°	0.74°

the membrane will cause corresponding differences in the tenacity with which the outside solution will adhere in the meshes of the membrane.

Only fertilized eggs were used in all the experiments reported in this paper. We may mention incidentally that we compared also the osmotic pressure of the fertilized with that of the unfertilized egg, but found no difference. This observation may be of interest in view of the striking differences found by Backman and Runnström on the fertilized and unfertilized egg of the frog.<sup>2</sup> Such differences do not exist in the eggs of *Fundulus*.

### III. THEORETICAL REMARKS.

We have found that the osmotic pressure of the juice pressed out from the egg of *Fundulus* with the aid of a Buchner press varies according to the concentration of the solutions in which the eggs had been kept previously. This was explained on the assumption that some of the water in which the eggs were washed or kept previous to the washing adhered to the meshes between the fibrils of which the membrane is composed. The question may be raised whether this influence of the concentration of the solution in which the eggs had been kept previously may not be explained on the assumption that the egg membrane is completely permeable to water and the substances dissolved in it. This assumption is contradicted by the experiments of Loeb on the floating of eggs and the duration of life of the embryo of *Fundulus* in solutions of high concentration.<sup>3</sup> When we put the eggs of *Fundulus* into a solution of 50 cc. of 3 M NaCl + 1 cc. 10/8 M CaCl<sub>2</sub> the eggs will float on such a solu-

<sup>2</sup> Backman, E. L., and Runnström, J., *Arch. f. d. ges. Physiol.*, 1912, cxliv, 287.

<sup>3</sup> Loeb, J., *Science*, 1912, xxxvi, 637; *Biochem. Ztschr.*, 1912, xlvii, 127.



tion, and the embryo will live for three days or longer, while the newly hatched embryos will die in such a solution in a few minutes. Moreover, Loeb has shown that it is impossible to adapt the fish to such high concentrations by gradually raising the osmotic pressure of the solutions.<sup>4</sup> These results and many similar ones are only intelligible on the assumption that the membrane of the egg of *Fundulus* is practically impermeable for water and salt, as long as the eggs are in physiologically balanced solutions, as was the case in the experiments reported in this paper. The results of the experiments reported in this paper also contradict the assumption that the outside solution diffuses into the egg. If this were the case, the osmotic pressure of the eggs in distilled water should become less and less the longer they remain in distilled water, which is not the case. The variations of osmotic pressure observed in our experiments are easily understood on the assumption that the osmotic pressure of the contents of the egg remains unchanged, but that traces of the solution in which the egg had been kept adhere for some time in the meshes of the fibrils forming the outer part of the membrane.

#### SUMMARY OF RESULTS.

1. It is made probable by experiment that the osmotic pressure of the contents of the egg of *Fundulus* corresponds to a freezing point depression of  $0.76^{\circ}$ .

2. The osmotic pressure of fertilized and unfertilized eggs of *Fundulus* is practically identical.

3. When the egg is washed or kept in solutions of different concentrations, the osmotic pressure of the juice pressed out of the egg varies somewhat with the outside contents. This is explained on the assumption that the membrane consists of fibrils and that some of the outside solution adheres to the meshes of the outer part of the membrane, without, however, entering into the egg.

4. This supports the idea, expressed in a previous paper by Loeb and Cattell, that when these eggs are put for some time into distilled water a layer of distilled water is formed inside the membrane which may act as a barrier to the diffusion of potassium into the egg.

<sup>4</sup> Loeb, *Biochem. Ztschr.*, 1913, liii, 391.

## AN ATTEMPT AT A PHYSICO-CHEMICAL EXPLANATION OF CERTAIN GROUPS OF FLUCTUATING VARIATION.

BY JACQUES LOEB AND MARY MITCHELL CHAMBERLAIN.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

### I.

There is a general tendency to visualize the factors which determine the hereditary characters as specific chemical compounds. If we wish to carry this view (with which we sympathize) beyond the limit of a vague statement, we must either try to establish the nature of these compounds by the methods of the organic chemist, or we must use the methods of general or physical chemistry and try to find numerical relations by which we can identify the quantities of the reacting masses or the ratio in which they combine. Attempts in this direction have been made by the suggestion of Loeb<sup>1</sup> that phenomena of growth belong in the group of auto-catalytic processes, and by T. B. Robertson's<sup>2</sup> and Ostwald's investigations supporting and enlarging this idea; by A. R. Moore's<sup>3</sup> attempt to show that in hybrids the velocity of development of the dominant character is slower than in the pure dominant breed; and by Loeb and Ewald's<sup>4</sup> proof that all the embryos of *Fundulus* have practically the same rate of heart beat at the same temperature. Since our new experiments are a sequence of this last mentioned paper, we may briefly discuss its contents.

<sup>1</sup> J. Loeb. Ueber den chemischen Character des Befruchtungsvorgangs. Roux's Vorträge und Ausätze, Leipzig, 1908. Biochem. Ztschr., 2, 34, 1906.

<sup>2</sup> T. B. Robertson. Roux's Archiv, 25, 581, 1908; 26, 108, 1908; 37, 497, 1913. Am. Jour. Physiol., 37, 1, 1915; Robertson and Wasteneys. Roux's Archiv, 37, 485, 1913; Wo. Ostwald. Ueber die zeitlichen Eigenschaften der Entwicklungsvorgänge, Leipzig, 1908.

<sup>3</sup> A. R. Moore. Roux's Archiv, 34, 168, 1912.

<sup>4</sup> J. Loeb and W. F. Ewald. Biochem. Ztschr., 58, 177, 1913.

C. G. Rogers<sup>5</sup> has shown that the heart beat of the embryo of *Fundulus* has a temperature coefficient of the order of the magnitude of a chemical reaction, i.e., that it practically doubles for an increase of temperature of 10°C. Loeb and Ewald found that the rate of heart beat is practically the same in each individual embryo (of a certain age) for a given temperature, varying only in very narrow limits; so that the rate of the heart beat of any of these embryos could be utilized as a thermometer. The authors explained this fact on the basis of general chemistry as follows: given a sufficient quantity of substrate the velocity of the reaction is in proportion to the mass of enzyme. If we suppose that the rate of the heart beat is determined by the velocity of an enzyme reaction—which supposition agrees with the temperature coefficient—we must conclude that all hearts of *Fundulus* embryos must have the same mass of enzyme, since they all beat at the same rate when the temperature is the same. If we consider the rate of heart beat of the *Fundulus* embryo a hereditary character—which is legitimate—we are forced to the conclusion that each embryo of *Fundulus* inherits practically the same mass of those enzymes which are responsible for the heart beat. The hereditary factor in this case must consist of material which determines the formation of a given mass of these enzymes, since the factors in the chromosomes are too small to carry the whole mass of the enzymes existing in the embryo or adult.

## II.

While the rate of heart beat is approximately the same in each egg (at the right age) and for the same temperature, we notice slight variations, the usual fluctuating variation. It occurred to us that this fluctuating variation might offer a chance for further testing the enzyme conception of the factors of certain hereditary characters. We selected, instead of the rate of heart beat, the velocity of cell division. Loeb<sup>6</sup> had shown in a former paper that the time from insemination to the first cell division in the egg of the sea urchin *Strongylocentrotus purpuratus* can be so sharply meas-

<sup>5</sup> C. G. Rogers. *Am. Jour. Physiol.*, 28, 81, 1911.

<sup>6</sup> J. Loeb. *Pflüger's Archiv*, 124, 411, 1908.

ured and is so nearly constant that it can be used for the establishment of a temperature coefficient and this was later confirmed by Loeb and Wasteneys<sup>7</sup> for the egg of *Arbacia*. Since the influence of temperature is again of the high order characteristic of chemical reactions, we may make the assumption that each egg carries a definite mass of one or more enzymes or catalysers which determine the rate of cell division. If we fertilize a mass of eggs of the same female of *Arbacia* and keep them at the same temperature, we find that they do not all begin to segment at the same time, and that there is an interval between the cell division of the first and last egg of the group. If we assume that the velocity of the cell division is determined by the mass of enzymes and the temperature, the fact that at  $t^{\circ}$  some eggs divide after 100, others after 101, 102, until, e. g., 113 minutes, we must conclude that this difference in time is the expression of a corresponding difference in the mass of enzymes in different eggs, those dividing in 100 minutes having a greater mass of enzymes than those dividing in 102, 103, etc., and 113 minutes; and that the mass of enzymes varies in inverse proportion to the time required for cell division at a given temperature. On this basis we should have to assume that the latitude of variation in the rate of cell division of a group of eggs is the expression of a corresponding variation in the mass of enzyme in the individual eggs. This idea can be put to a test with the aid of the temperature coefficient. If we call  $m$  the minimum mass of the enzyme responsible for the first cell division in the slowest eggs, then we shall find a certain greater percentage of eggs with the enzyme mass  $m + a_1$ , a still larger percentage with the mass  $m + a_2$ , and a small number with the mass  $m + a_n$  where  $m + a_n$  is the greatest mass of enzyme occurring in an egg. If the eggs with the mass  $m + a_n$  divide at the temperature  $t^{\circ}$  after 100 minutes, they will divide in about  $Q_{10} \times 100$  minutes at the temperature  $(t - 10)^{\circ}$ , where  $Q_{10}$  is the temperature coefficient for  $10^{\circ}\text{C.}$  at this point; the eggs with the smallest mass of enzyme  $m$ , which at  $t^{\circ}$  divide after 113' will divide at  $(t - 10)^{\circ}$  after  $Q_{10} \times 113$  minutes, since the temperature coefficient must be the same for both types of eggs. If we call the difference in the time of segmentation between the slowest and fastest

<sup>7</sup> J. Loeb and H. Wasteneys. *Biochem. Ztschr.*, 36, 345, 1911.

egg the *latitude of variation*, this latitude of variation should vary in direct proportion to the temperature coefficient for cell division if our theory is correct.

### III.

We will first give the temperature coefficient of cell division for the egg of *Arbacia* for different temperatures; i.e., the results of measurement of the time required from the moment of insemination to the moment when the first egg in the field was seen to divide. The eggs had been kept in a water bath with constant temperature,

TABLE I.

*Time in Minutes from Insemination to the Cell Division of the First Egg in Arbacia.*

TEMPERATURE	LOEB AND WASTENEYS	LOEB AND CHAMBERLAIN
<i>degrees</i>		
7.0	498.0	
8.0	410.0	411.0
9.0	308.0	297.5
10.0	217.0	208.5
11.0	175.0	175.0
12.0	147.0	148.0
13.0		129.0
14.0		116.0
15.0	100.0	100.0
16.0	85.5	
17.5	70.5	
18.0	68.0	68.0
19.0		65.0
20.0	56.0	56.0
21.0		53.3
22.0	47.0	46.0
23.0		45.5
24.0		42.0
25.0	40.0	39.5
26.0	33.5	
27.5	34.0	
30.0	33.0	
31.0	37.0	

and a little before the cell division was expected to occur (which time we knew from the former observations of Loeb and Wasteneys) the eggs were put into a watch glass of the temperature of the eggs and the exact time ascertained when the first egg of the lot underwent cell division. Table I gives these times according to Loeb and Wasteneys, and according to our own observations. The

reader will notice how closely both values agree.<sup>8</sup> Our values are the average of a number of determinations, which show only a negligible variation.

Beyond 31° no segmentation occurs. We tried no experiments on the latitude of variation beyond 25° or below 9°, since outside of these limits the segmentation is no longer entirely normal.

From the results of table 1 we compute the temperature coefficients for the time from insemination to the first appearance of cell division (table 2).

TABLE 2.

TEMPERATURE COEFFICIENT FOR	
8/18	$\frac{410}{68} = 6.0$
9/19	$\frac{297}{65} = 4.5$
10/20	$\frac{208.5}{56} = 3.7$
11/21	$\frac{175}{53.5} = 3.3$
12/22	$\frac{146}{46} = 3.2$
13/23	$\frac{129}{45.5} = 2.8$
14/24	$\frac{116}{42} = 2.8$
15/25	$\frac{100}{40} = 2.5$

In order to determine the latitude of variation of the time of segmentation—i. e., the interval between the time at which the first egg of a set begins to segment and the time when the last egg segments for a certain temperature, we proceeded as follows: The eggs were inseminated in sea water, and kept in a water bath at the desired temperature. The eggs remained in this water bath until about the time when the first segmentation was expected to occur. In the meantime, a second water bath was prepared on the stage of the microscope whose temperature was slightly below that of the desired temperature. This water bath contained the watch glass in which the segmentation of the eggs was to be observed. The watch

<sup>8</sup> The eggs were always used in the first hours after they had been removed from the animal. The time required for the first cell division was remarkably constant in different experiments. It is worth mentioning that such constancy is only possible when the temperature is kept constant.

glass had therefore the temperature at which the eggs were observed. The temperature of this water bath was also kept constant. When the temperature at which the latitude of variation was observed was very low and that of the air of the room was high a slight error crept in, in as much as the temperature of the water in

TABLE 3.  
*Latitude of Variation in Segmentation Time.*

NUMBER OF EGGS SEG- MENTED AFTER	TEMPERATURE					
	25°	15°	22°	22°	12°	12°
	Number of eggs in field					
<i>minutes</i>	117	127	116	126	116	100
1	3	1	1	2	4	3
2	12	6	8	24	15	5
3	34	15	21	49	26	8
4	68	34	33	85	40	10
5	107	44	85	95	51	12
6	10 eggs not fertilized	62	103	111	60	16
7		79	110	117	67	19
8		90	116	119	77	20
9		95		7 eggs not fertilized	80	24
10		100			80	28
11		109			88	32
12		18 eggs not fertilized			88	36
13					88	38
14					90	49
15					92	60
16					95	75
17						84
18					100	85
19					101	
20					105	85
21					105	95
22					106	96
23					108	
24					8 eggs not fertilized	
25						98 2 eggs not fertilized

the watch glass rose slightly during observation. This error made itself felt in that in the case of low temperatures the actual temperature was occasionally a trifle higher than intended. We shall come back to this point later on.

When the eggs had been put into the watch glass, a field with no less than 80 and often as many as 150 eggs was selected, and every minute the number of eggs which underwent cell division was

counted until the last egg had divided. Very often a small percentage of the eggs had remained unfertilized and these of course did not divide.<sup>9</sup> In table 3 we give a few examples of the actual measurements of the latitude of variation in the time required from the segmentation of the first to that of the last egg in a field.

As far as the irregularities in the first two minutes are concerned, they must probably be attributed to the fact that the entrance of the spermatozoa into the eggs occurred somewhat irregularly, the moment of insemination differing in various eggs within one or two minutes. Table 4 gives the latitude of variation, i. e., the difference in time between the segmentation of the last and that of the first egg in a field for different temperatures for all observations made. The averages appear in the last line.

TABLE 4.

*Difference in minutes between segmentation of first and last egg in a field at*

9°	10°	11°	12°	13°	14°	15°	18°	19°	20°	21°	22°	23°	24°	25°
50	39	25	22	20	17	13	12	14	10	8	8	9	7	5
49	40	26	20	18	19	12	11	13	10		7	7	9	5
47		27	22	(13)	16	12	13	11	9		8	9	7	5
64				19	18	12		12			9	8	7½	
60				20		14		12			7	8	9	
46				18		14		14			8	7	8	
				20		12					8			
						14					8			
						14					7			
											8			
											8			
Mean 52.6	39.5	26	22.5	19.2	17.5	13	12	12.5	9.6	8	7.8	8	8	5 Min.

This series illustrates the source of error to which we have already alluded, namely, that at low temperatures the times were liable to be too short when the outside temperature was very high. Thus the value 13 minutes for the temperature of 13° is unquestionably too low, and probably the values 46 and 47 for 9°C. are also too low. At the higher temperatures the values differ much less, since the temperatures approximate much more the room temperature.

We are now in a position to compare the expected with the observed result. The expected result is the series of temperature coefficients for the time from insemination to the time when the first

<sup>9</sup> When this number was great the material could not be used since in such cases the spermatozoa no longer entered the eggs simultaneously.



egg of the set begins to divide; the observed result is the series of temperature coefficients for the latitude of variation, i.e., the time which elapses between the segmentation of the first and last egg in a set. These two sets of coefficients should be identical and table 5 shows the degree of agreement.

A comparison shows that the temperature coefficients for the latitude of variation are practically identical with the temperature coefficients for cell division, and that where a noticeable difference

TABLE 5.  
*Temperature Coefficients for Latitude of Variation.*

TEMPERATURES	EXPECTED	FOUND
9/19	4.7	$\frac{52.6}{12.6} = 4.2$
10/20	3.8	$\frac{39.5}{10} = 3.9$
11/21	3.3	$\frac{26}{8} = 3.2$
12/22	3.1	$\frac{22.5}{7.8} = 2.8$
13/23	2.8	$\frac{19.2}{8} = 2.4$
14/24	2.8	$\frac{17.5}{8} = 2.3$
15/25	2.5	$\frac{13}{5} = 2.6$

exists it is always in the same direction, namely, the coefficients for the latitude of variation are a trifle too small. We can account for this on the basis of the deficiency in the method we have already discussed, namely that when the temperature of observation was low and that of the room high, the temperature in the watch glass may have risen slightly during the observations. Since in the determination of the temperature coefficient the value for the low temperature forms the numerator, it is obvious that the observed temperature coefficients are liable to be a little smaller than they would be without this error. We expect to test this idea next season.

#### *Theoretical Remarks.*

It was found in a previous investigation that the time which elapses from the moment of insemination to the moment of the

beginning of cell division in the egg of *Arbacia*, is a constant for a given temperature. On the basis of the enzyme theory this was to be explained on the assumption that the mass of ferments contained in the egg of the sea urchin responsible for this process is approximately constant in each individual egg. This would mean that the hereditary factor determining the rate of cell division consists in determiners for definite quantities of ferments. This idea was put to a test by applying it to the fluctuating variability of this process. While for a given temperature the eggs of *Arbacia* will always begin to segment at the same time, not all the eggs segment simultaneously. Assuming that those eggs which segment first have a greater mass of ferment than the others, fluctuating variability would in this case be due to differences in the mass of ferment in the different eggs of the same female. If this idea were correct, eggs with the maximum and with the minimum amount of ferment should differ in the rate of segmentation by an amount of time which would vary in direct proportion to the temperature coefficient for the process of segmentation. This theory was tested and it was found that the observed values agree very closely with the expected values; the slight variations found being in the direction of the possible source of error of the method of the experiments. These experiments support therefore the idea that the hereditary factor responsible for the rate of segmentation is a determiner for a given mass of certain ferments, and that fluctuating variability depends in this case upon slight but definite variations in the mass of those ferments in different eggs.

#### SUMMARY OF RESULTS.

1. It is shown that the temperature coefficient for the latitude of variation of the segmentation of the egg of *Arbacia* (i.e., the time between the segmentation of the first and last egg of a group fertilized at the same time) is practically identical with the temperature coefficient for segmentation.

2. It is shown that the fact is intelligible on the assumption that the fluctuating variation in this case is due to a variation in the mass of enzyme contained in the different eggs and supposed to be responsible for the rate of segmentation.

## CALCIUM IN PERMEABILITY AND IRRITABILITY.

By JACQUES LOEB.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, October 25, 1915.)

### I.

There has been a growing tendency to explain all phenomena of increased activity of the cell on the assumption of an increased permeability. Especially R. S. Lillie<sup>1</sup> has tried to harmonize many phenomena with this viewpoint, and it has been adopted by Bayliss<sup>2</sup> in a recent address. Thus it was assumed that the agencies of artificial parthenogenesis induce development by increasing the permeability of the egg; that the stimulus of nerve or muscle inducing muscular contraction is due to an increase in permeability induced by the stimulus; that the current of action (negative variation) is due to an increased permeability, and so on. Conversely it was assumed that narcosis is due to a diminution in permeability.

The idea that stimulation might be due to an increased permeability of the cell originated probably under the influence of the following facts. As is well known, muscular twitchings are produced when the muscle or the nerve is put into a pure NaCl solution, while the addition of Ca or Sr or Mg stops these twitchings. On the other hand, the writer found that for the egg of *Fundulus* pure NaCl in the concentration in which it occurs in sea water is toxic, while the addition of a small quantity of CaCl<sub>2</sub> (or any other salt with a bivalent metal) renders the NaCl harmless. In 1905 he suggested that this phenomenon might be explained on the assumption that in a pure NaCl solution the latter enters the membrane and

<sup>1</sup> Lillie, R. S., *Am. Jour. Physiol.*, 1909, xxiv, 14; 1911, xxviii, 197.

<sup>2</sup> Bayliss, W. M., *Science*, 1915, xlii, 509.

kills the egg, while the presence of a trace of a salt with a bivalent metal prevents this diffusion of NaCl into the egg.<sup>3</sup> The correctness of this explanation could later be demonstrated in the following way: When the egg of *Fundulus* is put into a mixture of 50 cc. 3 M NaCl + 1 cc. 10/8 M CaCl<sub>2</sub>, the embryo will live in this mixture for 3 days or longer, while if the newly hatched fish is put into such a solution it is killed almost instantly. When the egg is put into a pure 3 M NaCl solution the embryo dies within a few hours. These facts seem only intelligible on the assumption that the addition of a trace of CaCl<sub>2</sub> to the solution makes the egg impermeable for the NaCl, while without CaCl<sub>2</sub> the NaCl gradually diffuses into the egg.

It is, of course, natural to consider the possibility that the stimulating effect of a pure NaCl solution upon nerve or muscle is also due to an increase in permeability, while the CaCl<sub>2</sub> inhibits this increase in permeability.

The acceptance of such a view meets, however, with several difficulties. First, there is, for the present, a lack of direct proof for it, and second, it is apparently contradicted by certain facts, one of which may be mentioned. The center of the jellyfish *Polyorchis* will as a rule not contract in an isotonic NaCl solution, but will begin rhythmical contractions when a certain amount of CaCl<sub>2</sub> is added; but the contractions can also be called forth if instead of CaCl<sub>2</sub> some divalent or trivalent anion is added; *e.g.*, Na<sub>2</sub> tartrate or oxalate or Na<sub>3</sub> citrate.<sup>4</sup> On the basis of our present knowledge it is not probable that small quantities of Ca as well as of oxalate should both increase the permeability of the cell and counteract a diminution of permeability caused by NaCl.

An attempt has been made to connect the electromotive phenomena in living cells with assumed changes in the permeability of the membrane; but these attempts are not warranted.<sup>5</sup>

The writer has recently approached the possible connection of stimulation and permeability from a different viewpoint. Previous experiments had shown that the concentration of CaCl<sub>2</sub> (or of salts with bivalent cations) required for the antagonization of salts with

<sup>3</sup> Loeb, J., *Arch. f. d. ges. Physiol.*, 1905, cvii, 252.

<sup>4</sup> Loeb, *Jour. Biol. Chem.*, 1905-06, i, 427.

<sup>5</sup> Loeb, *Science*, 1915, xlii, 643.

univalent cation varies with the concentration of the latter. If the mechanism of antagonization is the same for phenomena of irritability as for permeability the ratio of  $\frac{C_{Na \text{ salt}}}{C_{Ca \text{ salt}}}$  should vary in the same way for both groups of phenomena with varying  $C_{Na}$ . This is not the case.

## II.

The eggs of *Fundulus* which normally develop in sea water develop also in distilled water and in solutions of higher osmotic pressure than sea water. If we put the newly fertilized eggs into pure NaCl solutions of different concentrations above  $3/8$  M NaCl the eggs will form embryos only if a minimal quantity of  $CaCl_2$  is added. This quantity varies with the concentration of NaCl. In a series of experiments that quantity of  $CaCl_2$  was ascertained which is required to permit 50 per cent of the eggs to form embryos in NaCl solutions of different concentrations. Table I gives the result.

TABLE I.

Concentration of NaCl.	Quantity of $M/16$ $CaCl_2$ required to allow 50 per cent of the eggs to form embryos.	Concentration of NaCl.	Quantity of $M/16$ $CaCl_2$ required to allow 50 per cent of the eggs to form embryos.
$3/8$ M	cc.	$9/8$ M	cc.
$4/8$ M	0.1	$10/8$ M	1.8-2.0
$5/8$ M	0.3	$11/8$ M	2.0-2.5
$6/8$ M	0.5	$12/8$ M	2.0(?)*
$7/8$ M	0.6	$13/8$ M	3.0-3.5
$8/8$ M	0.9		6.0
	1.2-1.4		

\* This value for Ca in an  $11/8$  M NaCl solution is presumably too low and due to an error.

In NaCl solutions of a concentration beyond  $13/8$  M it was not possible to cause 50 per cent of the eggs to form embryos, no matter how much Ca was added; in  $M/4$  NaCl, 50 per cent of the eggs could form embryos even without the addition of  $CaCl_2$ , which might possibly be understood on the assumption that the egg itself contains some  $CaCl_2$ . This might also explain why so little  $CaCl_2$  is needed for the development of the eggs in a  $3/8$  M NaCl solution.

It is obvious that the minimum amount of  $CaCl_2$  which must be

added increases much more rapidly than the concentration of NaCl. Thus if the concentration of NaCl varies in the ratio 1 : 2 : 3 (if we compare, *e.g.*, 4/8 M, 8/8 M, and 12/8 M NaCl), the values for  $\text{CaCl}_2$  increase in the ratio of 0.3 : 1.3 : 3.2, or, in other words, if we double the concentration of NaCl we must quadruple the amount of Ca added; and if we triple the concentration of NaCl we must add about ten times as much  $\text{CaCl}_2$ . *The value of Ca increases almost in the ratio of the square of the increase of the NaCl solution.*

### III.

We will compare with this the variation in the ratio  $\frac{C_{\text{Na}+\text{K}}}{C_{\text{Ca}+\text{Mg}}}$ ; *i.e.*, the ratio of the concentration of the chlorides of the monovalent over that of the bivalent cations in the sea water for a case of irritability. As material the newly hatched larvæ of a certain barnacle (*Balanus cburneus*) were used, which can stand wide variations in the concentration of the sea water.<sup>6</sup> These larvæ are strongly heliotropic and gather in dense clusters at the window-side or the opposite side of the dish. They are incessant swimmers and they rise to the surface of the water. They are able to live in sea water from the concentration of M/16 to 6/8 M.

When the larvæ are put into a pure solution of  $\text{NaCl} + \text{KCl}$  (in the proportions in which these two salts exist in the sea water) they will all fall to the bottom, unable to swim, though they may live for a number of hours in such a solution. If one salt with a bivalent cation is added, *e.g.*,  $\text{CaCl}_2$  or  $\text{MgCl}_2$  or  $\text{SrCl}_2$ , in sufficient quantity, they will rise to the surface but they cannot stay there very long; if, however, enough of a mixture of  $\text{CaCl}_2 + \text{MgCl}_2$  is added in the proportions in which these two cations exist in the sea water (1.5 atoms of Ca to 11.8 atoms of Mg) the larvæ will rise to the surface and remain there, gathering on the side by the window or away from it.

Experiments were made to ascertain the minimal quantity of  $\text{CaCl}_2 + \text{MgCl}_2$  required to allow all the animals to rise to the surface in different concentrations of  $\text{NaCl} + \text{KCl}$ . Table II gives the results.

<sup>6</sup> Loeb, *Proc. Nat. Acad. Sc.*, 1915, i, 439.

TABLE II.

No. of experiment.	Concentration of NaCl + KCl	Cc. of $\frac{3}{8}$ M $\text{CaCl}_2$ + $\text{MgCl}_2$ required.	Value of $\frac{\text{C}_{\text{Na}} + \text{K}}{\text{C}_{\text{Mg}} + \text{Ca}}$
I. ....	$\left\{ \begin{array}{l} \text{M}/16 \\ \text{M}/8 \end{array} \right.$	$\left\{ \begin{array}{l} 0.3 \\ 0.4-0.5 \end{array} \right.$	$\left\{ \begin{array}{l} 27.8 \\ 37.0 \end{array} \right.$
II. ....	$\left\{ \begin{array}{l} \text{M}/8 \\ \text{M}/4 \end{array} \right.$	$\left\{ \begin{array}{l} 0.5 \\ 0.9-1.0 \end{array} \right.$	$\left\{ \begin{array}{l} 33.3 \\ 35.1 \end{array} \right.$
III. ....	$\left\{ \begin{array}{l} 3/16 \text{ M} \\ 3/8 \text{ M} \end{array} \right.$	$\left\{ \begin{array}{l} 0.7 \\ 1.3 \end{array} \right.$	$\left\{ \begin{array}{l} 35.7 \\ 38.5 \end{array} \right.$
IV. ....	$\left\{ \begin{array}{l} \text{M}/8 \\ \text{M}/2 \end{array} \right.$	$\left\{ \begin{array}{l} 0.5 \\ 1.8-1.9 \end{array} \right.$	$\left\{ \begin{array}{l} 33.3 \\ 36.0 \end{array} \right.$
V. ....	$\left\{ \begin{array}{l} \text{M}/4 \\ \text{M}/2 \end{array} \right.$	$\left\{ \begin{array}{l} 0.8-0.9 \\ 1.6-1.7 \end{array} \right.$	$\left\{ \begin{array}{l} 39.2 \\ 40.3 \end{array} \right.$
VI. ....	$\left\{ \begin{array}{l} 5/16 \text{ M} \\ 5/8 \text{ M} \end{array} \right.$	$\left\{ \begin{array}{l} 0.9 \\ 1.7 \end{array} \right.$	$\left\{ \begin{array}{l} 46.3 \\ 49.0 \end{array} \right.$
VII. ....	$\left\{ \begin{array}{l} 3/16 \text{ M} \\ 6/8 \text{ M} \end{array} \right.$	$\left\{ \begin{array}{l} 0.6 \\ 2.4 \end{array} \right.$	$\left\{ \begin{array}{l} 41.7 \\ 41.7 \end{array} \right.$

Two experiments with concentrations of NaCl + KCl varying in the ratio of 1:2 or 1:4 were always made simultaneously. The permanent readings were taken a number of hours after the animals were put into the solutions. The result indicates that the ratio of  $\frac{\text{C}_{\text{Na}} + \text{K}}{\text{C}_{\text{Ca}} + \text{Mg}}$  remains very nearly constant with varying concentrations of  $\text{C}_{\text{Na}} + \text{K}$ . This relation corresponds to Weber's law, according to which the change in a stimulus which is just perceptible has a constant ratio to the original stimulus. Weber's law is the most general law in the realm of human sensations and therefore we need not be surprised at meeting such a law in this connection. This side of the problem was discussed in a former paper.<sup>7</sup>

It is, therefore, obvious that the ratio of  $\frac{\text{C}_{\text{Na}}}{\text{C}_{\text{Ca}}}$  for the phenomenon of irritability selected for discussion varies according to a different law than for the case of permeability. Our results, therefore, do not lend support to the idea that the rôle of calcium in phenomena of irritability is the same as in phenomena of permeability.

#### IV.

Not only in NaCl + KCl but also in NaCl + KCl +  $\text{MgCl}_2$  are the larvæ unable to rise for any length of time to the surface, while

<sup>7</sup> Loeb, *Proc. Nat. Acad. Sc.*, 1915, i, 439.

if we add some Ca the larvæ will do so. Experiments of the following kind were made. To 50 cc.  $M/2$  NaCl + KCl were added different quantities of  $MgCl_2$ , and it was ascertained how the quantity of  $CaCl_2$  necessary to cause the larvæ to rise and remain at the surface varied with the amount of Mg added. In former investigations the writer had shown that the swimming motions of the center of a jellyfish cannot continue in a mixture of Na + K + Mg, but that this effect of Mg can be promptly overcome by the addition of Ca;<sup>8</sup> and this antagonism between Ca and Mg was confirmed by Meltzer and Auer<sup>9</sup> in their experiments on mammals.

Our experiments consisted in adding to 50 cc.  $M/4$  or  $M/2$  NaCl + KCl (in the proportions in which these salts exist in sea water) varying quantities of  $3/8$  M  $MgCl_2$ . In such solutions the animals could swim for only a few minutes. If, however, some  $CaCl_2$  was added the animals could rise permanently to the surface and swim to or from the window-side of the dish. It was ascertained how much  $CaCl_2$  was required to cause the majority of the larvæ to rise. Table III gives the results.

TABLE III.

				Cc. of $M/16$ $CaCl_2$ necessary to induce the majority of the larvæ to swim in:	
				$M/2$ Na + K	$M/4$ Na + K
50 cc. NaCl + KCl		+ 0.75 cc. $3/8$ M $MgCl_2$	.....		0.2
"	1.5	"	.....	0.4	0.3
"	2.5	"	.....	0.4	0.4
"	5.0	"	.....	0.7-0.8	0.7-0.8
"	10.0	"	.....	1.6	1.6
"	15.0	"	.....	1.8	
"	20.0	"	.....	1.8	

In order to interpret these figures correctly we must remind the reader that we are dealing here with a combination of two antagonisms. The one is between the salts with univalent and bivalent metals. This antagonism is satisfied by merely adding enough  $MgCl_2$  to a mixture of NaCl + KCl. The reader will recall that in a mixture of NaCl + KCl +  $MgCl_2$  the larvæ will swim for a few minutes if enough  $MgCl_2$  is added. The second antagonism is

<sup>8</sup> Loeb, *Jour. Biol. Chem.*, 1905-06, i, 427.

<sup>9</sup> Meltzer, S. J., and Auer, J., *Am. Jour. Physiol.*, 1908, xxi, 400.



between  $\text{CaCl}_2$  and  $\text{MgCl}_2$ . With the addition of only  $\text{MgCl}_2$  the animals can swim but a short time; but if both  $\text{MgCl}_2$  and  $\text{CaCl}_2$  are added in the right concentration all the larvæ will swim permanently.

In the experiments in Table III enough  $\text{MgCl}_2$  was always present (with the exception of the first solution) so that the balance between salts with univalent and bivalent cations was established. What was lacking was the balance between Ca and Mg. The experiments of Table III therefore answer the question of how the concentration of Ca must change if the concentration of Mg changes. If we consider only the concentrations of Mg between 2.5 and 10.0 cc.  $\frac{3}{8}$  M Mg, we find again that the  $C_{\text{Ca}}$  must vary directly in proportion to  $C_{\text{Mg}}$ , which again is Weber's law. Thus if the  $\text{MgCl}_2$  added varies from 2.5:5:10.0 cc., *i.e.*, in the ratio of 1:2:4, the quantities of  $\text{CaCl}_2$  required are 0.4:0.8:1.6 cc., which is also the ratio of 1:2:4.

The normal concentration of Mg is about 6.0 cc. in 50 cc. of solution. Hence, as long as the concentration of Mg is neither excessively high nor low, the law of proportion nearly holds. Only when the concentration of Mg is very low or excessively high do we find deviations from this law; but this is a peculiarity which we find in all other cases of Weber's law.

It agrees also with our statement that it makes no difference whether the 50 cc.  $\text{NaCl} + \text{KCl}$  are present in  $\frac{M}{4}$  or  $\frac{M}{2}$  solutions, since we are dealing here only with the antagonism between Ca and Mg.

Experiments in which the original mixture was  $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ , and where the quantity of  $\text{MgCl}_2$  required to induce most or all of the larvæ to swim was ascertained, gave no results which could be utilized for quantitative measurements, for the reason that it is impossible to find a sharp end-point which could serve as a standard of measurements. In a mixture of  $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ , when the concentration of  $\text{CaCl}_2$  is normal or below normal, the larvæ lie a long time on the bottom of the dish, finally some will rise and swim to the light. The number which will swim will be increased by the addition of  $\text{MgCl}_2$ , but not in a way which permits quantitative experiments.

## SUMMARY.

The variation of the amount of Ca, or of Ca + Mg, required to antagonize various concentrations of NaCl, or of NaCl + KCl, was investigated for a case where the antagonism concerned the permeability, and for a case where it concerned irritability. It was found that in the case of irritability the Ca required varied in direct proportion to the change in the concentration of NaCl (Weber's law), while in the case of permeability the concentration of Ca required for antagonism varied approximately with the square of the ratio of the concentration of NaCl.

## THE SALTS REQUIRED FOR THE DEVELOPMENT OF INSECTS.

By JACQUES LOEB.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, October 8, 1915.)

### I.

The writer reported recently that it is possible to raise the banana fly on a sterilized liquid medium consisting of water, one or two sugars (grape sugar and cane sugar), one ammonium salt (*e.g.*, ammonium tartrate), and some inorganic salts.<sup>1</sup> Such a mixture is a well known culture medium for certain microorganisms; *e.g.*, yeast cells, which are capable of synthesizing their proteins and other complicated organic compounds from ammonium salts. He left the question undecided whether or not microorganisms (either in symbiosis with the fly or carried with it to the culture medium) acted as an intermediate in this synthesis, and he is not yet ready to give an answer. He has since tried to find out which inorganic salts are required for the completion of the life cycle of the fly. This question is interesting for the following reason. For microorganisms the indispensable ions are, as a rule, K, Mg, PO<sub>4</sub>, and SO<sub>4</sub>, while very often neither NaCl nor CaCl<sub>2</sub> is required. In 1900 the writer called attention to the fact that for the rhythmical contractions of the jellyfish, *Gonionemus*, NaCl was required and that for the continuation of these contractions NaCl and CaCl<sub>2</sub> were required in certain proportions.<sup>2</sup> Lingle confirmed this for the heart beat of the tortoise,<sup>3</sup> and Overton showed later that the nerve and muscle of the frog lose their irritability reversibly if they are kept

<sup>1</sup> Loeb, J., *Science*, 1915, xli, 169.

<sup>2</sup> Loeb, *Am. Jour. Physiol.*, 1900, iii, 383.

<sup>3</sup> Lingle, D. J., *Am. Jour. Physiol.*, 1902-03, viii, 75.

for some time in a sugar solution, while their irritability is preserved if a slight amount of NaCl is added to the sugar solution.

The banana fly possesses a high degree of motility, and it was therefore of interest to know which inorganic salts would be required to raise a number of successive generations possessing normal activity.

The experiments were carried on in a platinum vessel. The nutritive solutions consisted of the following mixture:

	<i>gm.</i>
Grape sugar .....	0.5
Cane sugar .....	0.5
Ammonium tartrate .....	0.1
Citric acid <sup>4</sup> .....	0.05
K <sub>2</sub> HPO <sub>4</sub> .....	0.005
MgSO <sub>4</sub> .....	0.005
H <sub>2</sub> O .....	3 cc.

All the substances used were the purest that could be obtained.

The solution was put into a platinum vessel. Into this vessel was put a basket of silver netting which just touched the upper surface of the solution. The flies were put into this basket, which allowed them to lay the eggs on the surface of the nutritive solution but prevented the flies from falling into the liquid and drowning. The platinum vessel was put into a glass cylinder about 10 cm. high which was closed with absorbent cotton. Before the beginning of the experiment the whole was sterilized by heating in an autoclave to 120° for one hour. At first three pairs of flies were put into the vessel, left there for four days in order to lay their eggs, and then quickly removed. After this the flies raised in the platinum dish were used for propagation in the manner described.

Thus far, five successive generations of flies have been raised under these conditions in the platinum vessel. The motility of the flies is perfectly normal. The experiments show that without any other NaCl or CaCl<sub>2</sub> than that which may appear as impurities in the chemicals used, five and probably indefinite generations of flies can be raised.

<sup>4</sup> The citric acid was added to keep the solution acid and to exclude the development of bacteria as much as possible.

The only salts added were  $K_2HPO_4$  and  $MgSO_4$ . Numerous control experiments made in glass vessels showed that without either the addition of K or  $PO_4$  no larvæ can be raised. When Na was substituted for K no flies could be raised. I am not sure whether Mg and  $SO_4$  are as indispensable as K and  $PO_4$ , since in  $K_2HPO_4$  alone occasionally a fly developed. It is certain, however, that the addition of  $MgSO_4$  greatly increased the number of flies raised.

As far as the evidence from these experiments goes we can, therefore, say that in these flies the muscular activity is possible either without any Na or Ca or with only such traces as appear in the form of impurities in the chemically pure substances used in these experiments; while K as well as  $PO_4$ , and also  $SO_4$ , and Mg, must be added to the culture medium in appreciable quantity.

We intend to repeat these experiments with substances which shall be absolutely free from Na and Ca.

## II.

The experiments show that as highly organized an animal as the banana fly can be raised on a culture medium as simple as that required for certain microorganisms.

As far as the writer is aware it is generally assumed that the evolution of higher animals could only have taken place after green plants had come into existence, since the latter serve directly or indirectly as food for the animals. While this is generally true for our present fauna, the possibility is not excluded that an evolution of animals as highly specialized as insects might have taken place independently of the existence of green plants.

The investigations of Winogradski<sup>5</sup> and of Godlewski on nitrite and nitrate bacteria seem to have made it certain that these organisms are capable of forming carbohydrates from carbon dioxide (or possibly other carbon compounds in the air) independently of light; and the same may be true for certain other microorganisms. Microorganisms of this type might, therefore, suffice to furnish the carbo-

<sup>5</sup> Winogradski, S., *Handb. d. tech. Mykol.*, 1904, iii, 162. See also Beijerinck, M. W., *Folia Microbiologica*, 1914, iii, 91.

hydrates necessary for the development of other microorganisms which require sugars for their growth. Even if we assume that in our experiments yeast cells<sup>6</sup> or other microorganisms acted as intermediates in the building up of proteins for the fly (which is quite possible), it is obvious that an evolution of animals as complicated as the banana fly (which usually lives on plant food) might have been possible without the existence of chlorophyll, provided that Winogradski's conclusions are correct. •

<sup>6</sup> Guyénot, E. (*Compt. rend. Soc. de biol.*, 1913, lxx, pt. i, 178), has shown that yeast can serve as food for *Drosophila*, and it has been stated that in Germany yeast has become a general food for higher animals.

## THE ACTION OF ETHYLHYDROCUPREIN (OPTOCHIN) ON TYPE STRAINS OF PNEUMOCOCCI IN VITRO AND IN VIVO, AND ON SOME OTHER MICRO- ORGANISMS IN VITRO.

By HENRY F. MOORE, M.B., B.Ch., B.A.O.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, May 25, 1915.)

In 1911 Morgenroth and Levy (1) introduced the drug ethylhydrocuprein (optochin), a derivative of hydroquinine, in the treatment of experimental pneumococcic infection. Subsequent tests have, as a rule, shown the compound to possess a parasiticial effect on the pneumococci. In the experiments so far reported, different strains of pneumococci, chosen more or less at hazard, were studied; no attempt, however, has been made to investigate the action of the drug on type strains of the various groups of pneumococci. It seemed of interest to investigate the action of the drug from this viewpoint.

Neufeld (2) showed that strains of pneumococci differed among themselves in respect to their immunity reactions (protective bodies). The investigations of Cole (3) and of Dochez and Gillespie (4) have resulted in a serological classification of the pneumococci. It has been shown by these authors that the pneumococci can be divided into at least four groups. The organisms of Groups I and II are specific in their immunity reactions; that is, an immune serum produced against any member of Group I has a specific protective action against, and a specific agglutinative action on, any member of Group I, but has no effect on any members of Groups II, III, or IV. Similarly, an antiserum to any member of Group II will react in the same way with all members of that group, but not with any members of Groups I, III, or IV. In Group III are included all members of the *Pneumococcus mucosus* type. To Group IV belong all pneumococci not belonging to Groups I, II, or III. An antiserum to any member of Group IV exhibits protective and agglutinative reactions with the strain used for its production, but in no instance with any other member of Group IV or

with any member of the other three groups. Lister (5) has arrived at a somewhat similar classification from opsonic studies.

#### EXPERIMENTAL.

The pneumococci used in the present study were representative of these four serological groups and were obtained from the Hospital of The Rockefeller Institute, with the exception of the micro-organism designated as South Africa (9), which was obtained from the South African Institute for Medical Research; this organism has been placed in a fifth group by Lister (5) as a result of his opsonic studies. All strains of pneumococci used in the present study were isolated from cases of human pneumonia, except those called I Les and II White, which were obtained from cases of pneumococcal meningitis by lumbar puncture.

#### *The Action of Ethylhydrocuprein in Vitro.*

Wright (6) has shown that the action of ethylhydrocuprein hydrochloride on pneumococci *in vitro* is only slightly lessened by the presence of serum, while with antiseptics such as lysol and cresol, under similar conditions, the contrary is the case. Tugendreich and Russo (7) have shown that the hydrochloride of the drug in a concentration of 1 in 16,000 kills the pneumococcus *in vitro* in three hours at room temperature, and that it is much more potent in this respect than the hydrochlorides of the homologous compounds: isopropyl, isobutyl, and isoamylhydrocuprein; this superiority is even more marked over quinine and hydroquinine. In the present study the action of ethylhydrocuprein hydrochloride on type strains of the different groups of pneumococci was worked out and compared with that on other microorganisms under similar conditions.

*Technique.*—A solution of the drug was made in broth and sterilized by boiling or filtration through a Berkefeld filter. Various dilutions of this stock solution were made; to 2 cc. of each dilution in a test-tube 0.1 cc. of a 24 hour broth culture was added and the tubes were incubated for 18 hours at 37° C. At the end of this period the tubes were examined, and the results, as regards growth or inhibition of growth, noted; a large loopful of the two





lowest dilutions showing growth macroscopically, and of all tubes showing no growth, was in each case plated in about 12 cc. of blood agar and incubated. The concentration of the drug was thus enormously diluted. The plates were examined at the end of 24 hours and again at the end of 48 hours. Thus, one could differentiate between an inhibitory effect of the drug on the growth of the microorganism and actual death. Some typical results are given in Table I.

*Explanation of Tables.*—In the top row the Roman numeral stands for the serological group to which the strain of pneumococcus belongs; a letter and number in brackets, if present, denote the particular strain; the Arabic number represents the number of animal passages of the strain and the exponent the number of cultivations on artificial medium since the last animal passage. In the remaining two rows each number represents the greatest dilution in which the growth of the corresponding microorganism was inhibited or killed, as the case may be. In the case of the pneumococci the range of dilutions of the drug examined was as follows: I in 10,000; I in 20,000; I in 50,000; I in 100,000; I in 500,000; I in 1,000,000; and I in 10,000,000.

From the results given in Table I it is seen that ethylhydrocuprein hydrochloride causes in very high dilutions, *in vitro*, an inhibition of growth, and death of the pneumococcus, the latter occurring, generally speaking, in somewhat lower dilutions; and that no constant or considerable differences are seen in these effects on typical representatives of the four groups of pneumococci.

It was thought of interest to compare the action on pneumococci with that on other organisms, more especially streptococci. The results are set forth in Table II.

### *Description of the Microorganisms Mentioned in Table II.*

Streptococcus I was a non-hemolytic streptococcus cultivated by Dr. Beattie from a case of rheumatic fever. The culture used was obtained from the Bacteriological Department of the Museum of Natural History, New York.

Streptococci 5 and 7 were two different strains of non-hemolytic streptococci obtained from the Pathological Department of Mt. Sinai Hospital, New York. They were cultivated from the blood of patients suffering from subacute endocarditis.

TABLE II.

*The Action in Vitro of Ethylhydrocuprein Hydrochloride on Microorganisms Other than Pneumococci.*

Micro-organism.	<i>Micrococcus car- searthalis</i> .	<i>Strepto- coccus mucosus</i> .	<i>Strepto- coccus 1.</i>	<i>Strepto- coccus 5.</i>	<i>Strepto- coccus 7.</i>	<i>Strepto- coccus 8.</i>	<i>Strepto- coccus 59.</i>	<i>Strepto- coccus R.</i>	<i>Strepto- coccus C.</i>	<i>B. coli com- munis</i> .	<i>B. typhosus</i> .	<i>B. para- typhosus B.</i>	<i>Staphylo- coccus albus</i> .	<i>B. Fried- lander</i> .
Highest dilu- tion causing inhibition of growth	10,000	20,000	Not in- hibited in 10,000	20,000	10,000	10,000	10,000	20,000	10,000	Not in- hibited in 10,000	Not in- hibited in 10,000	Not in- hibited in 10,000	Not in- hibited in 10,000	10,000
Highest dilu- tion causing death	Not killed in 10,000	Not killed in 10,000	Not killed in 10,000	20,000	Not killed in 10,000	Not killed in 10,000	Not killed in 10,000	Not killed in 10,000	Not killed in 10,000	—	—	—	—	Not killed in 10,000

TABLE III.\*  
The Action in Vitro of Quinine Hydrochloride.

Microorganism.	1 to 5.	1138.	111A66.124.	IVM.4.	Strepto- coccus mucosus.	Micro- coccus catarrhalis.	Strepto- coccus I.	B. coli communis.	Staphylo- coccus albus.
Highest dilution causing inhibition of growth.....	50,000	50,000	50,000	50,000	5,000	5,000	5,000	1,000	1,000
Highest dilution causing death.....	5,000	10,000	5,000	10,000	1,000	5,000	2,000	1,000	1,000

\* The first four organisms are representatives of the four groups of pneumococci.

*Streptococcus* 8 was a laboratory strain of hemolytic streptococcus.

*Streptococcus* 59 was a green-producing, non-hemolytic streptococcus cultivated by Dr. H. F. Swift from the blood of a patient suffering from rheumatic pericarditis.

None of these strains had a capsule, fermented inulin, or was bile-soluble.

The *Streptococcus mucosus* was a diplococcus growing in short chains; it showed, with His's stain, a well defined capsule in the body fluids of an infected animal; it was not bile-soluble; it was pathogenic for mice, causing a sticky exudate in the peritoneal cavity; it did not ferment inulin; on cultivation for a few passages on blood agar the growth, which was previously moist on solid media, became dry in character.

The streptococcus strains called R and C were isolated from normal sputum and were mucous in type; that is, they gave a mucoid growth on blood agar and possessed a capsule; these qualities were, however, lost after a few passages on artificial media. Neither was bile-soluble, and neither fermented inulin.

From Table II it is seen that the action, if any, of ethylhydrocuprein, in the dilutions examined, on the microorganism mentioned therein was considerably less than that on the pneumococci.

The action *in vitro* of the hydrochloride of quinine (from which ethylhydrocuprein is derived) on pneumococci and on some other microorganisms is shown in Table III. It is seen therefrom that, while the action of quinine hydrochloride is greater on the pneumococci than on the other bacteria, this action is far less in the former case than that of optochin.

#### *The Action of Ethylhydrocuprein (Optochin Base) on Representatives of the Four Groups of Pneumococci in Vivo.*

Owing to the fact that certain biochemical relationships (such as bile solubility) exist between trypanosomes (8) and spirilla (9), on the one hand, and the pneumococci (10) alone among the *Coccae*, on the other, Morgenroth (1) investigated the action on the pneumococci of that group of compounds which has given results in the therapy of trypanosomal and spirillar infections; namely, quinine and its derivatives.

Morgenroth and Levy showed that ethylhydrocuprein (a derivative of hydroquinine) exerted a considerable protective action, and a certain degree of curative action (1) on experimental pneumococcal infection in the mouse. Later Gutmann (11) and Morgenroth and Kaufmann (12) reported experiments in which this protective action on several strains of pneumococci was shown. Levy (13) studied the curative effect of the drug on pneumococcal infection of the mouse by a strain of *Pneumococcus mucosus*, an interval of from two to six hours having been allowed to elapse between the intraperitoneal infection and the first

administration of the drug. The dosage of the infection does not seem to have been accurately measured in Levy's experiments, nor do the results seem to have been checked by autopsy; an effect of the drug, however, on the infection can be seen; of 16 mice, 10 survived up to the 8th day, when the observation was discontinued. This work is summarized by Rosenthal (14).

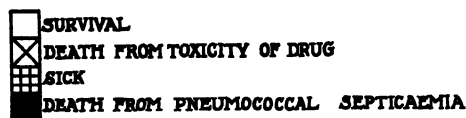
So far, the strains studied in this connection were chosen more or less at haphazard, no attempt having been made to study strains of pneumococci in relation to classification. In the following experiments typical representatives of the four groups of pneumococci were used.

*Technique.*—A 2 per cent solution of the free base ethylhydrocuprein (optochin base) was made in sterile olive oil by rubbing up in a mortar and standing the product so obtained in the incubator at 37° C. over night. Mice of 18 grams' weight and upwards were used. The infection was given in a constant volume of 1 cc. of broth intraperitoneally. The treatment consisted of 0.5 cc. of the 2 per cent optochin base solution in oil (calculated in every case) per 20 grams of mouse given under the skin of the back immediately after the infection; this was followed by an equal dose on the second day of the experiment, and by 0.4 cc. per 20 grams of mouse on the third and fourth days. The mice were observed for a period of from 10 to 14 days. An autopsy was performed on every mouse that succumbed (whether a treated animal or a control), and the heart's blood, and peritoneal fluid if necessary, were examined in a smear preparation, Gram's stain and His's capsule stain being used. If no Gram-positive and capsule-bearing bacteria were found in the smear by these methods, cultures were made on defibrinated rabbit blood agar with abundant inoculation, incubated for 24 hours at 37° C., and then examined.

In tabulating the results the graphic method recommended by Morgenroth is employed. In the text-figures each square denotes one experimental animal; a black square indicates death from pneumococcal septicemia; a white square, survival; an oblique cross denotes that the animal died, but that the examination of the heart's blood was negative, in which instance death was apparently caused by the toxicity of the drug, the infecting pneumococci having been first killed off by its action;<sup>1</sup> and, finally, a square subdivided into

<sup>1</sup> Morgenroth pointed out that the toxic dose for mice is not far removed from the curative dose.

six smaller ones denotes that the animal was sick at the time of observation (Text-fig. 1).

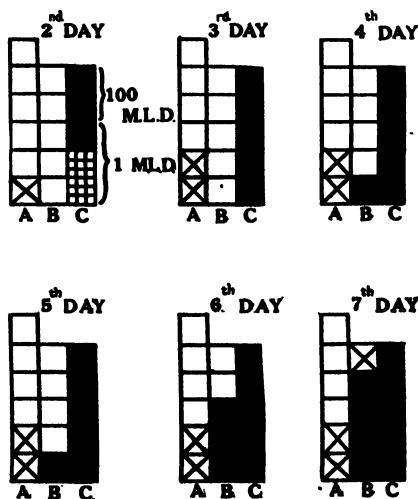


TEXT-FIG. 1. Explanation of the method used in the text-figures.

The condition of the animals is first given in the diagram on the second day of the experiment; that is, 24 hours after the infection and first treatment; consequently, the first diagram in each experiment is marked 2d day.

The minimum lethal dose of the infecting microorganism was in each case determined for forty-eight hours unless otherwise stated; in all cases that dose was taken as the m. l. d. which was with certainty fatal within the time limit stated for several mice. In case of doubt reestimations were made. With this statement it is unnecessary to give the estimations in detail.

Throughout the observations the mice were kept at about 75° F.<sup>2</sup>

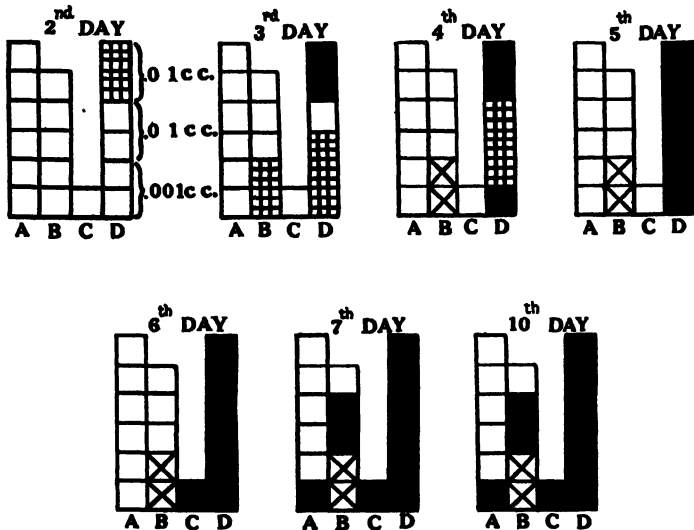


TEXT-FIG. 2. Experiment 1. Strain I 106.<sup>4</sup> The m.l.d. was 0.000,000,001 cc. (48 hrs.). The infecting doses used: 10 m.l.d. (Column A); 1,000 m.l.d. (Column B). Column C, controls (untreated).

<sup>2</sup> The toxicity of the drug is said to be greater when the animals are kept exposed to cold.

*Comment on Experiment 1.*—(Text-fig. 2.) Of the 6 treated animals infected with 10 times the m. l. d., 2 died of toxicity, the heart's blood at autopsy being sterile; while the remaining 4 animals remained permanently well; of those infected with 1,000 times the m. l. d. and treated, 1 died of toxicity (showing sterile heart's blood at autopsy), and the remaining 4 of pneumococcal septicemia; the last mentioned 4 animals, however, survived much longer than the untreated controls.

This microorganism was of extraordinarily high virulence for mice, it having had 106 animal passages. The m. l. d. above stated was regularly fatal and showed 6 to 14 colonies when plated.

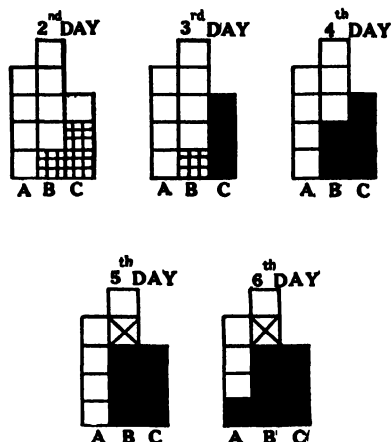


TEXT-FIG. 3. Experiment 2. Strain I (B42)<sub>16</sub>. The m.l.d. was 0.001 cc. The infecting doses used: 10 m.l.d. (Column A); 100 m.l.d. (Column B); 500 m.l.d. (Column C). Column D, controls (untreated).

*Comment on Experiment 2.*—(Text-fig. 3.) This strain was relatively much less virulent than the previous one. A dose of 0.001 cc. or 0.01 cc. was always fatal within 4 to 5 days, but sometimes the animals infected with the larger of these doses survived those infected with the smaller by a day. In view of the protracted course of the infection in this case the time limit for the m. l. d. was judged as 5 days. Of those animals injected with 10 times the m. l. d. only 1 died of pneumococcal septicemia, all the others

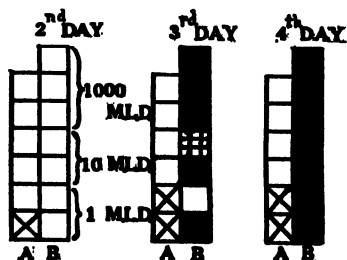


surviving. Of those injected with 100 times the m. l. d. 1 survived; 2 died of toxicity; and 2 of pneumococcal septicemia, but later than the controls.



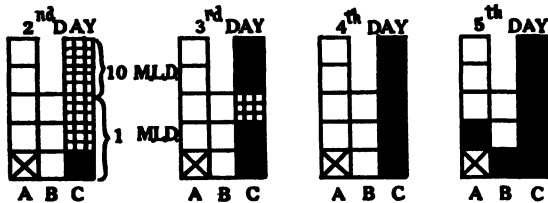
TEXT-FIG. 4. Experiment 3. Strain II 34<sup>19</sup>. The m.l.d. was 0.000,000,001 cc. (48 hrs.). The infecting doses: 10 m.l.d. (Column A); 100 m.l.d. (Column B). Column C, controls (untreated).

*Comment on Experiment 3.*—(Text-fig. 4.) Of the 4 animals infected with 10 times the m. l. d. and treated, 1 died on the 6th day of pneumococcal septicemia and 3 survived. Of the 5 infected with 100 times the m. l. d. and treated, 3 died of pneumococcal septicemia and 1 survived; the remaining animal died of toxicity. On plating the m. l. d., 6 to 12 colonies resulted. This strain was one of very high virulence, notwithstanding the fact that it had undergone nineteen artificial cultivations since the last animal passage.



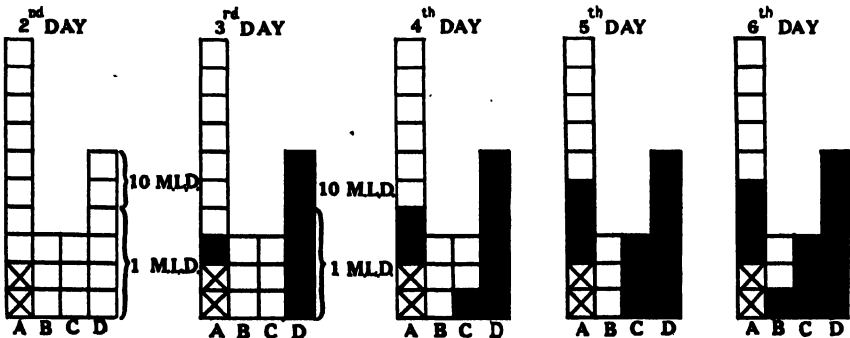
TEXT-FIG. 5. Experiment 4. Strain II (B21) 3<sup>2</sup>. The m.l.d. was 0.0,000,001 cc. (fatal in 4 days). Dose used: 1,000 m.l.d. (Column A). Column B, controls (untreated).

*Comment on Experiment 4.*—(Text-fig. 5.) If the m. l. d. is judged to kill in 4 days, 4 mice out of 6 infected with 1,000 times the m. l. d. survived the controls permanently. Of the 2 given in Text-fig. 5 as dying of toxicity the heart's blood of 1 was sterile, and a Gram-negative bacillus was recovered from that of the other, but no pneumococci.



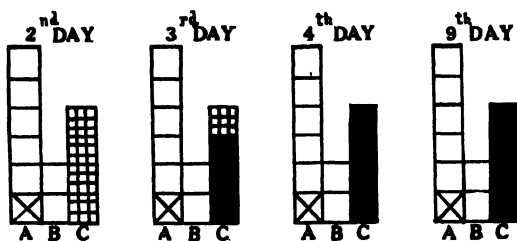
TEXT-FIG. 6. Experiment 4 a. Strain II (B21) 214. The m.l.d. was 0.01 cc. (48 hrs.). Doses used: 1 m.l.d. (Column A); 50 m.l.d. (Column B). Column C, controls (untreated).

*Comment on Experiment 4 a.*—(Text-fig. 6.) In this experiment 1 animal represented in Column A (1 m. l. d.) and 1 in Column B (50 times the m. l. d.) died on the 8th and 10th days, respectively, the heart's blood showing on examination a Gram-negative bacillus but no pneumococci. As we were troubled with mouse typhoid at this time, this was probably the cause of death. 5 animals survived, 2 died of pneumococcal septicemia, and 1 of toxicity.



TEXT-FIG. 7. Experiment 5. Strain III (A66) 128. The m.l.d. was 0.000,000,001 cc. (48 hrs.). Doses used: 10 m.l.d. (Column A); 100 m.l.d. (Column B); 1,000 m.l.d. (Column C). Column D, controls (untreated).

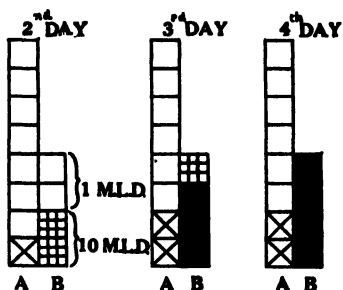
*Comment on Experiment 5.*—(Text-fig. 7.) The Strain III (A 66) 12<sup>6</sup> was a very virulent one. The m. l. d., 0.000,000,001 cc. of a 24 hour broth culture, when plated on blood agar, showed on incubation at least 15 typical colonies. The animal represented by the upper segment of Column C was found almost completely eaten by its fellows on the 6th day; no autopsy could be done, consequently the cause of death is somewhat doubtful. After the 6th day no further deaths occurred in this series. 5 out of 10 animals injected with 10 times the m. l. d. survived; 3 died of pneumococcal infection, and 2 of toxicity (giving a sterile heart's blood). Of 3 injected with 100 times the m. l. d. 2 survived, and the remaining animal succumbed to the infecting pneumococci. Of those injected with 1,000 times the m. l. d. none survived.



TEXT-FIG. 8. Experiment 6. Strain III (E22) 12<sup>6</sup>. The m.l.d. (0.000,001 cc.) was fatal in 48 hrs. 0.00,000,001 cc. of a broth culture was observed to be regularly fatal within four days; the m.l.d. was not, however, at the time of this experiment determined for a longer period than 48 hrs.; consequently the dose of 0.000,001 cc. was probably considerably greater than the m.l.d. for a mouse. Column A, 1 m.l.d. (0.000,001 cc. (48 hrs.)). Column B, 10 m.l.d. (0.00,001 cc. (48 hrs.)). Column C, controls, 1 m.l.d. (untreated).

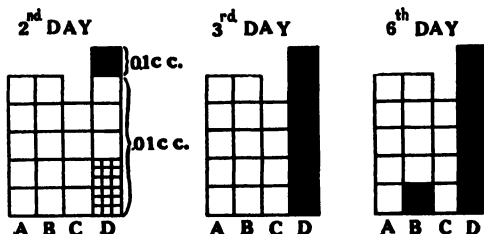
*Comment on Experiment 6.*—(Text-fig. 8.) Among the treated animals no death occurred up to the 10th day when observation was discontinued. 1 animal of the series died of toxicity, and none of pneumococcal septicemia.<sup>3</sup>

<sup>3</sup> Further experience with this strain has shown it to be more susceptible to the action of ethylhydrocuprein *in vivo* than any other strain of pneumococcus examined by us. We have been able with the drug to protect against 10,000 times the m.l.d.



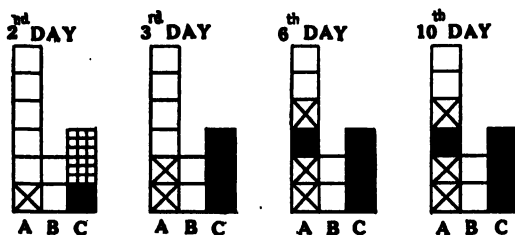
TEXT-FIG. 9. Experiment 7. Strain IV M.4<sup>3</sup>. The m.l.d. was 0.00,000,001 cc. of a 24 hr. broth culture. Dose used: 10 m.l.d. (Column A). Column B, controls (untreated).

*Comment on Experiment 7.*—(Text-fig. 9.) No further deaths occurred after the 4th day. Of 8 animals injected with 10 times the m. l. d. and treated, 2 died of toxicity and 6 survived. This was an unusually virulent strain of Group IV. The virulence, however, rapidly fell off on artificial cultivation.



TEXT-FIG. 10. Experiment 8. Strain South Africa (9).5<sup>2</sup>. The m.l.d. was 0.01 cc. Doses used: 10 m.l.d. (Column A); 20 m.l.d. (Column B); 50 m.l.d. (Column C). Column D, controls (untreated).

*Comment on Experiment 8.*—(Text-fig. 10.) Of the mice infected with 10 times the m. l. d. and treated all survived; of those infected with 20 times the m. l. d. and treated only 1 died of pneumococcal septicemia, all the others surviving; all those infected with 50 times the m. l. d. and treated survived. The controls infected with 1 m. l. d. all died within 48 hours.



TEXT-FIG. 11. Experiment 9. Strain IV (S 10)<sup>319</sup>. The m.l.d. was 0.01 cc. (48 hrs.). Dose used: 10 m.l.d. (Column A); 50 m.l.d. (Column B). Column C, controls, 1 m.l.d. (untreated).

*Comment on Experiment 9.*—(Text-fig. 11.) Of the 5 animals infected with 1 m. l. d. and treated 2 survived, and 3 died of toxicity. From the heart's blood of one of these latter a Gram-negative bacillus was recovered. One died of pneumococcal septicemia. Both the animals infected with 50 times the m. l. d. and treated recovered.

#### DISCUSSION.

The results of the present study agree, in the main, with those of the other workers mentioned. We have not had in our experiments such a large percentage of cures as is claimed by Morgenroth, namely 90 to 100 per cent; nor have we seen any definite protective action of optochin *in vivo* with an amount of infection greater than 1,000 times the m. l. d. of a highly virulent strain.<sup>4</sup> Moreover, the greater the virulence of the strain (by passage through mice) the greater was the difficulty in protecting against increasing multiples of the m. l. d. Constant results are difficult to obtain in the mouse, owing to the repeated injections apparently causing the animals a considerable degree of traumatism and consequently rendering them liable to intercurrent troubles. This was true of an epidemic of mouse typhoid which tended to obscure the results. The relative toxicity of the drug for mice is another factor which tends to render results not quite clear. Olive oil, the vehicle in which the drug is given, does not undergo absorption for a week or more, and the disturbance to the tissues caused by the repeated injections of this inert body seems to afford

<sup>4</sup> See page 262, footnote 3.

a suitable nidus for the growth of bacteria which may kill the animal before the observation is concluded. It seems likely that the detrimental factors, toxicity of the drug, the traumatism inflicted by the injections, and the effect of the infection itself, summate in their effects and thus render the probability of the animal surviving less likely, although its body may have been completely sterilized from pneumococcal infection. The effect of ethylhydrocuprein *in vitro* and *in vivo* on the pneumococcus is considerable and specific and is seen on type strains of all four groups of pneumococci.

From the text-figures it is seen that, of 85 mice infected with 100 times the m. l. d. or less and treated, 15 mice, or 17.6 per cent, died of pneumococcal septicemia; 13 mice, or 15.2 per cent, died of toxicity of the drug or some obscure cause, the heart's blood being sterile at autopsy (the corresponding controls invariably died of pneumococcal septicemia); 56 mice, or 66.8 per cent, survived. Of these 85 treated mice, 69, or 81 per cent, either recovered or died of causes other than pneumococcal septicemia; such, for example, as the toxicity of the drug.

#### CONCLUSIONS.

1. Ethylhydrocuprein hydrochloride in very high dilution inhibits the growth of, and in 18 hours kills, representatives of all four groups of pneumococci *in vitro*. The killing effect is generally seen in somewhat lower dilutions than the inhibiting effect. No constant or considerable difference is seen in these actions on representatives of the four groups of the pneumococci. The action of ethylhydrocuprein hydrochloride on the pneumococci *in vitro* is so strongly specific that it may possibly be used as a test for a true pneumococcus.

2. The inhibitory or killing effects of ethylhydrocuprein hydrochloride *in vitro* on bacteria other than pneumococci are slight or absent. The effects are greater on streptococci than on any other organisms examined, but are still much less than on the pneumococci. This action distinguishes between the streptococcus group, including *Streptococcus mucosus* sometimes found in normal

mouths, on the one hand, and the true pneumococcus (including *Pneumococcus mucosus*), on the other.

3. Quinine hydrochloride inhibits the growth of, and kills the pneumococcus *in vitro*; much stronger concentrations, however, are necessary than in the case of ethylhydrocuprein. This effect of quinine hydrochloride is also seen on other organisms, but in a less degree.

4. Ethylhydrocuprein (optochin base) has a well marked protective action against experimental pneumococcal infection in mice in the case of type strains of all four groups of pneumococci; this protective action may be efficient against many multiples of the minimum lethal dose.

#### BIBLIOGRAPHY.

1. Morgenroth, J., and Levy, R., *Berl. klin. Wchnschr.*, 1911, xlviii, 1560, 1979.
2. Neufeld, F., and Haendel, L., *Ztschr. f. Immunitätsforsch., Orig.*, 1909, iii, 159; *Berl. klin. Wchnschr.*, 1912, xlix, 680; *Arb. a. d. k. Gsndtsamte*, 1910, xxxiv, 169; in Kolle, W., and von Wassermann, A., *Handbuch der pathogenen Microorganismen*, 2d edition, Jena, 1913, iv, 513.
3. Cole, R., *Arch. Int. Med.*, 1914, xiv, 56.
4. Dochez, A. R., and Gillespie, L. J., *Jour. Am. Med. Assn.*, 1913, lxi, 727.
5. Lister, F. S., Specific Serological Reactions with Pneumococci from Different Sources, *The South African Institute for Medical Research [Publications]*, Dec. 22, 1913.
6. Wright, A. E., *Lancet*, 1912, ii, 1633, 1701.
7. Tugendreich, J., and Russo, C., *Ztschr. f. Immunitätsforsch., Orig.*, 1913, xix, 156.
8. Schilling, *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1902, xxxi, 452.
9. Neufeld, F., and von Prowazek, *Arb. a. d. k. Gsndtsamte*, 1907, xxv, 494.
10. Neufeld, F., *Ztschr. f. Hyg. u. Infektionskrankh.*, 1900, xxxiv, 454.
11. Gutmann, L., *Ztschr. f. Immunitätsforsch., Orig.*, 1912, xv, 625.
12. Morgenroth, J., and Kaufmann, M., *Centralbl. f. Bakteriol., Ref.*, 1912, liv, Supplement, 69.
13. Levy, R., *Berl. klin. Wchnschr.*, 1912, xlix, 2486.
14. Rosenthal, F., *Ztschr. f. Chemotherap., Ref.*, 1912, i, 1149.

## THE EFFECT OF IRRITATION ON THE PERMEABILITY OF THE MENINGES FOR SALVARSAN.

By EDGAR STILLMAN, M.D., AND HOMER F. SWIFT, M.D.

*(From the Hospital of The Rockefeller Institute for Medical Research,  
New York.)*

(Received for publication, June 9, 1915.)

One of the explanations offered for the beneficial effects of intraspinal injections in the treatment of syphilis of the central nervous system is that there may thus be induced an increased permeability of the meninges following their irritation by the injected substance. The purpose of this investigation was to determine whether the intraspinal, or rather subdural, injection of various substances used in intraspinal treatment would increase the amount of arsenic in the cord and brain of animals which received intravenous injections of salvarsan at the same time.

Sicard and Reilly (1) described a method for increasing the permeability of the brain substance for drugs by trephining the skull and injecting 5 cc. of a 0.5 per cent solution of sodium chloride under the dura with a fine hypodermic needle. They showed that in the cadaver a similar amount of India ink injected subdurally was distributed over an area 8 or 10 cm. in diameter, and suggested from this experimental evidence that two subdural injections of saline, *i. e.*, one in each temporo-frontal region, would be sufficient to increase the permeability of a large part of the cerebrum for salvarsan introduced intravenously. They also state that they have injected 0.1 mg. of cyanide of mercury subdurally in the frontal region without harmful effect, and on the following day given salvarsan or neosalvarsan intravenously to the same patient. The cyanide of mercury was used simply to increase the permeability of the tissues. Viton (2) states that after a slight chemical irritation the meninges are rendered more permeable, and he used for this purpose a preparation suggested by Sicard (3) which consists of cyanide of mercury 0.1 mg., and novocain 0.015 gm. in 2 cc. of a 0.5 per cent saline solution. The intraspinal injections were made at intervals of 1 month, mercury or salvarsan being given in the usual way. He states that with this method of treatment the irritative symptoms of tabes are much relieved. Tinel and Leroide (4) injected several drops of a 1 per cent solution of sodium nucleinate into the fourth ventricles of rabbits and immediately afterwards gave the animals 10 cg. of neosalvarsan intravenously. One hour later they again punctured the fourth ventricles and obtained 4 cc. of fibrinous fluid,



which contained 0.008 mg. of arsenic. Fluid from the fourth ventricles of control animals contained no arsenic. The fibrinous fluid was evidence of a much more intense irritation than is usually produced by subdural injections in patients, so the increase in permeability which these authors demonstrated cannot be applied to bedside treatment.

The intraspinal injection of normal salt solution, or serum, does produce, however, a temporary irritation of the meninges, as is shown both clinically by pains, and by examination of the cerebrospinal fluid a few hours after such an injection, when several hundred cells per cmm. may be found. It is reasonable to suppose that this irritation might increase the permeability of the tissues, and that salvarsan circulating in the blood would be deposited in larger amounts in the tissues contiguous to the irritated meninges, than when the meninges are in their normal impermeable condition.

### *Methods.*

To determine whether a demonstrable increased deposition of arsenic actually does occur, the following experiments were performed. Cats were selected as the experimental animals, for the following reasons: Intravenous injections can be easily given into the marginal vein of the ear, and there is a fairly large subdural cistern surrounding the cauda equina, so that subdural injections in the cat are similar in nature to those in man in that the injecting needle is not brought into direct contact with the cord. In rabbits the cord extends so low that it is impossible to introduce a needle under the dura and inject a solution without injuring the cord. In the experiments under discussion it was necessary that the nervous tissue be injured in no other way than that possibly resulting from the presence of the substance injected. The intraspinal injections in the cats were made in the following manner: The animals were etherized, and the dura was exposed by laminectomy of the two lower lumbar vertebræ. A fine curved hollow needle attached to a syringe was introduced through the dura, and cerebrospinal fluid was aspirated into the syringe, which was then detached, and another syringe containing the solution to be injected was attached to the needle, and the solution slowly injected. The injections were always preceded by aspiration of cerebrospinal fluid, to be sure that the needle was properly placed. All the animals were

injected intravenously with salvarsan in alkaline solution in the proportion of 0.05 of a gram per kilo of body weight. Some of the animals received the intravenous injections one hour before the intraspinal treatment, and in others the order was reversed, so that at the time of intravenous injection the meninges would be already in a state of irritation. On the following day the animals were exsanguinated by opening the jugular veins and carotid arteries, in order to remove as much arsenic-containing blood from the tissues as possible. The cord and brain were immediately removed from the body and the brain was divided into three portions: cerebrum, cerebellum, and midbrain, pons, and medulla; the cord was separated from the medulla, and all the specimens were dried separately. The dried tissue was powdered, thoroughly mixed, and weighed, and duplicates of each specimen were analyzed quantitatively for arsenic.

The analyses were made by a special method devised for the purpose by Vinograd (5). This method depends for its accuracy upon the principle of oxidizing the tissues with small amounts of arsenic-free nitric acid, at 260° C. in a sealed glass bomb. The bomb is then opened, sulphuric acid added, the nitric acid driven off by heating, and the sulphuric acid-arsenic mixture quantitated for arsenic by Sanger and Black's modification of Gutzeit's method. All reagents and utensils were carefully tested for arsenic before using. The standard scales for comparison were made with both arsenious acid and salvarsan. In the results here presented the figures are given in fractions of mg. of salvarsan per gram of dried tissue.

#### RESULTS.

The operative procedures, substances injected, time relations, and results of the analyses are given in Table I. Four control animals were first treated. Two of them had only intravenous injections of salvarsan. One of these killed one and one half hours later showed no more arsenic in its cerebrospinal axis than one killed after eighteen hours. The comparatively slight neurotropic action of salvarsan demonstrated by Ullmann (6) and by Stühmer (7) probably explains this similarity in arsenic content at such different intervals after treatment. In the other two controls the operative procedure, laminectomy and withdrawal of cerebrospinal fluid, was shown to

have no effect. The effect of irritation with the following substances was studied: isotonic salt solution; 50 per cent cat serum diluted with isotonic salt solution; pure cat serum; salvarsanized cat serum obtained by treating cats with 0.05 of a gram of salvarsan per kilo of body weight, bleeding one hour later, separating the serum, and heating to 56° C. This serum contained between 0.015 and 0.025 mg. of salvarsan per cc. of serum. It was injected in 50 per cent dilution and also undiluted. A mixture of cyanide of mercury, novocain, and 0.5 per cent saline, similar to that used by Sicard and by Viton, was also injected.

As a rule, the cords of the ten animals which received intraspinal injections contained no more arsenic than the four controls. The two exceptions are Animals H 104 and H 106, which received intraspinal injections of normal cat serum in a 50 per cent dilution. There are two possible explanations for this variation. First, the 50 per cent serum may have been more irritating than the other substances injected; or, second, the animals were not so completely exsanguinated. We are inclined to attribute the increased amount of arsenic in the cords of these animals to incomplete exsanguination, for in both animals the cerebrum and cerebellum contained considerably more arsenic than the average. Furthermore, if the 50 per cent serum were the important factor, one would expect that the cords of Cats H 110 and H 112, which received intraspinal injections of 50 per cent dilution of salvarsanized serum, would show a similar increase in arsenic, but in these animals both the cords and brains showed the average arsenic content. Upon first thought, one would expect that the cerebrospinal axis of the animals that received subdural injections of salvarsanized serum would contain more arsenic than the animals injected with normal serum or mercury. The rapid diffusion of the small amount of arsenic in the serum through the cerebrospinal fluid, and the rapid excretion of substances from the cerebrospinal fluid into the blood stream, probably explain the fact that the cords and brains of animals which received salvarsanized serum intraspinally contained no more arsenic than the average. Both of these factors have been conclusively demonstrated by Dandy and Blackfan (8). Hall (9) has also shown that the cerebrospinal fluid of patients who received

TABLE I.

Animal	Weight	Operative procedure.	Substance injected intraspinally.	Time relation of intra-spinal injection (I.S.) to intravenous injection (I.V.).	Killed after intravenous injection.	Salvarsan per gm. of dried tissue.				Remarks.
						Cerebrum.	Cerebellum.	Midbrain, pons, medulla.	Spinal cord.	
	gm.				hrs.	mg.	mg.	mg.	mg.	
H 115	3,000	None	None		1½	0.025	0.025	0.025	0.017	
H 113	2,560	"	"		18	0.017 (N)*	Traces	0.025	0.025 (N)*	
H 105	3,000	Laminectomy	"		19	0.037	0.037	0.025	0.025	
H 107	1,080	Laminectomy and 1 cc. cerebrospinal fluid withdrawn	"	1 hr. before I.V.	18	0.025	0.025	0.017	0.017	
H 116	3,100	Laminectomy and subdural injection	1 cc. 0.9% sodium chloride solution	"	17	0.017	Traces	0.025	Traces	
H 104	2,800	"	1 cc. 50% normal cat serum	" before "	13	0.037	0.037	0.025	0.075	
H 106	1,900	"	"	" after "	17	0.075	0.075	0.025	0.037 (N)*	
H 109	1,550	"	1 cc. 100% normal cat serum	" before "	16	0.025	0.017	0.025	0.025	
H 108	2,050	"	"	" after "	16	0.025	Traces	0.025	0.025	Weakness in both hind legs day after operation.
H 112	2,050	"	1 cc. 50% salvarsanized cat serum	" before "	15	0.017	0.025	0.017	0.025	
H 110	2,250	"	"	" after "	19	0.017	0.017	Traces	0.025	Weakness in right hind leg day after operation.
H 111	1,670	"	1 cc. 100% salvarsanized cat serum	"	19	0.025	Traces	0.017	0.025	
H 117	2,500	"	Mercury cyanide solution	" before "	19	0.025	0.017 (N)*	0.025	0.025	Weakness in both hind legs day after operation.
H 118	2,850	"	"	" after "	16	0.017	Traces	Traces	0.025	

\* (N), duplicate lost. All results are in duplicate unless otherwise noted.

intraspinal injections of neosalvarsan often contains no arsenic twenty-four hours after the injection.

If irritation alone were the important factor in determining the deposition of arsenic in the nervous tissue, the cords, which were more exposed to the irritating effect of the substances injected than the brains, should have shown a higher arsenic concentration. The average arsenic content of the cord and of the cerebrum was practically the same; *i. e.*, 0.029 mg. for the former, and 0.028 mg. for the latter. The cords of the three animals, H 108, H 112, and H 113, which showed clinical evidences of cord or cauda equina injury did not contain more arsenic than the cords of animals which showed no evidence of local lesions. Nor did the more irritating mercury solutions increase the arsenic in the cords or brains of the animals which received it. There was no difference between the animals that received intraspinal injections an hour before the intravenous injections of salvarsan, and those that first received the salvarsan intravenously.

#### CONCLUSIONS.

The subdural injection of normal salt solution, normal serum, serum salvarsanized *in vivo* or weak solutions of cyanide of mercury does not demonstrably increase the permeability of the spinal cord or brain for salvarsan which is circulating in the blood at the time of the subdural injection.

#### BIBLIOGRAPHY.

1. Sicard, J. A., and Reilly, *Bull. et mém. Soc. méd. d. hôp. de Paris*, 1913, xxxvi, series 3, 861.
2. Viton, J. J., *Semana med.*, 1913, xx, 1393; abstracted in *Jour. Am. Med. Assn.*, 1914, lxii, 582.
3. Sicard, *Bull. et mém. Soc. méd. d. hôp. de Paris*, 1913, xxxvi, series 3, 681.
4. Tinel, J., and Leroide, J., *Compt. rend. Soc. de biol.*, 1913, lxxiv, 1073.
5. Vinograd, M., *Jour. Am. Chem. Soc.*, 1914, xxxvi, 1548.
6. Ullmann, K., *Wien. klin. Wchnschr.*, 1913, xxvi, 216.
7. Stühmer, A., *Arch. f. Dermat. u. Syph.*, 1914, cxx, 589.
8. Dandy, W. E., and Blackfan, K. D., *Am. Jour. Dis. Child.*, 1914, viii, 406.
9. Hall, G. W., *Jour. Am. Med. Assn.*, 1915, lxiv, 1384.

## CHANGES IN THE ELECTROCARDIOGRAMS ACCOMPANYING EXPERIMENTAL CHANGES IN RABBITS' HEARTS.

By FRANCIS R. FRASER, M.B.

*(From the Hospital of The Rockefeller Institute for Medical Research.)*

PLATES 37 TO 40.

(Received for publication, June 14, 1915.)

Differences in electrical potential are developed in the structures of the heart during the processes of its action. At any instant of time the waves of the electrocardiogram are the resultant of a number of potential differences developed by the synchronous activities of the cardiac structures. It has been sufficiently demonstrated that the P wave is associated with activity of the auricles, and the Q, R, S, T, and U waves with activity of the ventricles. Clement (1), Erfmann (2), and Lewis (3) have recently shown that the Q, R, and S waves are associated with the spread of the excitation wave from the auriculoventricular junction to the ventricles and throughout the ventricular muscle mass. It is unknown what ventricular structures are associated with the individual waves. It is also unknown whether the direction of the potential difference responsible for a given wave corresponds to any anatomical structure. On theoretical grounds Einthoven (4) and Waller (5) have shown that from the relative sizes of a given wave in the three leads of the human electrocardiogram it is possible to calculate the angle which the direction of potential difference responsible for the wave makes with the anatomical axis of the body. Theoretically then, alterations in the position of the heart in the body that change the direction of the potential difference relatively to the axis of the body will change the size and direction of the waves. Again, if without any change in the position of the heart, alterations occur within the heart so that the distribution of the muscle mass is changed, as for

instance by a change in the thickness of the wall of one ventricle relatively to the wall of the other, or by a deviation of the septum to one side or the other, changes in the resultant potential difference would result with corresponding changes in the size and direction of the waves. Other factors are involved in determining the potential differences and character of the waves. These include the direction and rate of travel of the wave of excitation within the conducting structures and the muscle mass.

On account of these considerations it is generally recognized that when changes in the electrocardiogram occur they do so only as the result of definite causes. Instances of such changes are observed in hypertrophy of the human heart.

In patients with hypertrophy of the left ventricle, the R wave is large in Lead I and small or absent in Lead III, while the S wave is large in Lead III and small or absent in Lead I; and in patients with hypertrophy of the right ventricle, the R wave is small or absent in Lead I and large in Lead III, while the S wave is large in Lead I and small or absent in Lead III. These changes correspond to the changes expected theoretically, if in hypertrophy the direction of potential difference responsible for the Q, R, S group is turned to the left or to the right respectively. Other changes have been observed in the course of acute and chronic illnesses; as, for instance, alterations in the size of all or of some of the waves, splitting of waves, and lengthening of the time occupied by a wave or group of waves. These changes are often transitory and so must depend on causes other than increased muscle mass.

The work reported here was undertaken originally in the hope of correlating certain changes in the electrocardiogram with definite changes in the heart.

#### *Method and Material.*

For this purpose it was necessary to record changes in the heart during life. For observations on the position, size, and shape of the heart, x-ray plates were made. It was impossible to determine changes in the distribution of the muscle during life. For this and for the condition of the cardiac structures it was necessary to depend upon the examinations made after death. The postmortem examinations were satisfactory for these purposes only in the animals that

died or were killed at the time when the electrocardiographic changes were observed. After death the hearts were divided as recommended by Müller (6), and the chambers weighed separately. It was not found advisable to separate the two auricles and auricular septum from each other. Histological examinations were then made.

Rabbits were used in all the experiments. Full growth rabbits were chosen so that changes consequent on growth could be excluded, as it was found necessary to continue the observations in some cases for a period of six months or more.

In all, 33 rabbits were used, of which 4 were kept as controls. The changes were produced by repeated intravenous injection of: (a) spartein sulphate and adrenalin hydrochloride in 7 animals; (b) adrenalin hydrochloride alone in 4; (c) diphtheria toxin in 4; (d) suspensions of living streptococci in 14 animals.<sup>1</sup>

Spartein sulphate and adrenalin hydrochloride were used in the doses recommended by Fleisher and Loeb (7), and the injections were repeated at intervals of three or more weeks. Adrenalin alone was given in increasing doses every two days commencing with 0.1 or 0.2 cc. of 1:1,000 solution for twenty or more doses. Diphtheria toxin was given in varying doses and these were repeated if no definite effect was seen. The streptococci which were injected were isolated from the joints and blood of cases of acute rheumatism, from a tonsillar abscess, a liver abscess, and an abscess about the root of a tooth. Some were hemolytic, others of the *Streptococcus viridans* type. The injections were repeated, as a rule, in increasing doses until effects on the animal or electrocardiographic changes were seen.

The roentgenograms were made with the anticathode of the tube at a distance of 30 inches from the lower end of the sternum. The rabbit was stretched out on its back on a board, in which a gap had been cut so that the plate was in contact with the back of the animal.

Before injection, control electrocardiograms were made with the animal on the right side and on the left side, as well as on its back, so that possible alterations due to changes in the position of the heart

<sup>1</sup> The animals injected with streptococci were from a series of experiments undertaken with Dr. Homer F. Swift. The organisms were isolated and the injections made by him.



in the body could be estimated. In only four instances were changes in the size of the waves of more than 1 mm. noted with change of position.

The large Edelmann model of the string galvanometer was employed. On all occasions three leads were recorded. In most cases Lead I (right foreleg and left foreleg) was unsatisfactory, as the waves were very small; and Lead III (left foreleg and left hindleg) was, as a rule, so nearly identical with Lead II (right foreleg and left hindleg) that it has been found sufficient to consider Lead II only.

The resistance of the animal was estimated by the Wheatstone bridge and telephone method, and the string standardized with a constant total resistance in the circuit. The electrocardiograms were made with a resistance added to bring the total resistance in the circuit to a constant value. The deflection times of the different strings employed varied and the times occupied by some of the waves of the rabbits' electrocardiograms are so short that the size of the wave was affected by the variations in deflection time. It has been necessary, therefore, to discard all records in which the deflection time of the string varied from that of the control curves. Records in which the standardization of the string was not satisfactory have also been discarded. More rapid strings than those available might have shown greater changes than have been observed by the methods employed, but such changes as have been recorded would have been shown and shown better by an improvement in the methods.

#### RESULTS.

The changes in the electrocardiograms to be described are those involving the Q, R, S group only. Changes in the P and T waves also occurred but were so small, except in a very few instances, that accurate comparisons were impossible.

In the course of the observations on the 7 animals injected with spartein and adrenalin the roentgenograms in three animals showed that extensive dilatations to the right or to both sides developed in the course of a few days. Dilatations to the left were accompanied by decrease in the size of the R waves and increase in the size of the S waves; the one instance of marked dilatation to the right by

increased R wave and diminished S wave; while dilatation to both sides caused little or no change. These acute changes disappeared as quickly as they developed. Of the other 4 animals, 2 died after the first injection, and 2 after the second, and before significant changes developed. After repeated injections and the resultant acute changes, these 3 animals showed a moderate gradual enlargement to the left associated with decrease in the size of the R waves and increase in the S waves.

Figs. 1 and 3 represent the changes that occurred in Rabbit 218 E. Fig. 1 shows the size of the heart at the beginning, and Fig. 3 at the conclusion of the experiments, and they demonstrated an enlargement to the left side. Figs. 2 and 4 show the corresponding electrocardiographic changes, and Fig. 5 shows the extreme but transitory electrocardiographic changes obtained in this animal. Unfortunately no roentgenogram was obtained on the same day as this curve.

Figs. 6 and 8 represent the right-sided enlargement that occurred in Rabbit 211 E, and Figs. 7 and 9 represent the change in the electrocardiograms. Figs. 10 and 12 represent the extreme change obtained in Rabbit 278 E and Figs. 11 and 13 give the corresponding electrocardiograms. The enlargement in this case was to both sides while the electrocardiogram was altered but little. In Table I the changes in the waves and in the size of the heart throughout the observations in Rabbit 278 E are tabulated. It will be seen that following the third injection the electrocardiographic changes indicated an enlargement to the left side, but no roentgenogram was obtained on that date to correspond to this. Similar changes occurred after the last injection. Daily x-ray observations at this time showed an enlargement to the left.

Of the 4 animals treated by repeated injection of adrenalin alone, one, 106 B, showed an acute enlargement to the left accompanied by increased R wave and increased S wave, but died after the 4th injection, while the others showed slight gradual increase to the left with similar electrocardiographic changes.

Of the animals injected with adrenalin, or spartein and adrenalin, those that showed marked distress immediately after injection developed acute changes, while in those that showed little immediate

TABLE I.\*

Rabbit 278 E.

Received 6 Injections of Spartein Sulphate 0.012 Gm. per Kilo, and Adrenalin Hydrochloride 0.2 Cc. 1:1,000 (Parke, Davis and Co.). Total Resistance in Circuit 13,500 Ohms throughout Observations. String Standardized with Resistance in Circuit of 8,700 Ohms.

Date.	Injection.	Rate.	Deflection time.	Size of waves.			Size of heart.	Remarks.
				Q	R	S		
			sec.	mm.	mm.	mm.	mm.	
Dec. 2, 1913		267.3			+3.5	-1.0		On back.
" " "		266.4	0.020		+3.0	-1.0		On right side.
" " "		272.7			+2.5	-1.0		On left side.
" 4, "							28 x 23	
" 8, "	1							
" 12, "		246.7	"		+2.5	-1.0		
" 19, "		281.8	"		+2.0	-1.0		
" 30, "		284.3	0.025		+2.5	-3.0		
Jan. 7, 1914		267.5	"		+1.5	-1.5		
" " "	2							
" 12, "		293.7	"		+2.5	-2.0		
" 20, "		216.9	0.015		+2.0	-1.0		
" 22, "							32 x 28	
" 26, "		235.2	0.018		+2.5	-2.0		
" " "	3							
" 29, "		259.2	0.025	- ?	+ ?	-1.5		
Feb. 3, "		261.8	"			-3.5		
" 10, "		274.5	"		+0.5	-2.0		
" 26, "		267.1	0.020		+1.5	-2.0		
Mar. 5, "							38 x 32	
" 17, "		235.4	"		+2.0	-2.0		
" " "	4							
" 23, "		289.1	"		+3.0	-1.0		
" 25, "		273.1	"		+2.0	-0.5		
" 30, "		266.9	"		+2.0	-1.0		
Apr. 6, "							53 x 44	Enlarged to both sides.
" 7, "		261.7	"		+2.0	-1.0		
" " "	5							
" 10, "		255.6	"		+2.0	-1.0		
" 15, "							39 x 33	
" 16, "		230.8	"		+2.0	-2.0		
" 27, "							39 x 31	
" 30, "		233.8	"		+2.5	-2.0		
" " "	6							
May 1, "		254.2	"		+2.0	-1.5	38 x 32	
" 4, "		253.9	"		+1.5	-2.5	37 x 32	
" 5, "		240.0	"		+1.0	-2.0	36 x 33	
" 6, "							35 x 31	
" 7, "		238.4	"		+1.0	-3.0	35 x 30	
" 8, "		244.8	"		+1.0	-3.0	42 x 38	Enlarged to left mainly.
" 11, "		258.2	"		+1.0	-3.5	36 x 29	
" 13, "		268.2	"		+1.0	-2.5	34 x 26	
" 24, "		267.0	"		+1.0	-3.0		
" 25, "							34 x 25	
June 4, "		201.1	"		+2.0	-1.5		
" 5, "		234.8	"		+2.0	-1.5	33 x 25	Enlarged to left mainly.

\* The figures in this table and in Table II denoting the size of the heart were obtained by measuring and expressing in mm. the long axis of the heart shadow and the greatest width at right angles to this.

reaction to the injections only slight changes developed in the size of the heart or in the electrocardiograms. In all cases the heart sounds became accentuated and reduplicated and in four cases systolic murmurs developed, but except for restlessness and increased excitability, the animals did not appear to be distressed after recovering from the dyspnea consequent on the injections. This dyspnea never continued for more than a few minutes.

The three animals injected with fatal doses of diphtheria toxin showed slowing of the heart rate before death. Not only did the auricular rate decrease but the auriculoventricular conduction time lengthened, and the time occupied by each phase of the cycle increased. For example, in Rabbit 103 B the heart rate changed from 261.7 to 177.6, the P-R time from 0.07 of a second to 0.10 of a second, the time occupied by the Q, R, S waves from 0.02 to 0.07 of a second, and that occupied by the T wave from 0.12 to 0.16 of a second. Rabbit 105 B, which received three non-fatal doses, showed by x-ray slight enlargement of the heart downwards and to the left with increase in the S wave and decrease in the R wave of the electrocardiogram. Histologically the cases that received fatal doses showed marked degeneration and fatty changes throughout the heart muscle and similar changes in the auriculoventricular bundle. The electrocardiographic changes are illustrated in Figs. 14 and 15 from Rabbit 102 B.

No constant changes were seen in the fourteen rabbits that received intravenous injection of streptococci. Lesions resulting from these injections in structures other than the heart occurred but will not be discussed here. Rabbit 115 B was injected with a streptococcus obtained from the blood of a case of acute articular rheumatism. Slowing of the auricular rate occurred and slight lengthening of the auriculoventricular interval and of the Q, R, S time. The changes were similar to those seen in the cases injected with diphtheria toxin. Premature ectopic ventricular contractions also occurred before death. Histologically the heart muscle showed diffuse degenerative changes and numerous necrotic foci. Rabbit 118 B injected with a streptococcus isolated from a tonsillar abscess also developed premature ectopic ventricular contractions, but no focal lesions were found and the muscle showed but slight degenera-

tive changes. Figs. 16 and 17 illustrate the electrocardiographic changes in this animal. Rabbit 119 B injected with the same organism showed lengthening of the Q, R, S period. In the heart there were numerous areas of focal necrosis and degeneration of the muscle. Rabbit 108 B, injected with a *Streptococcus viridans* obtained at death from the heart's blood of a case of acute rheumatic carditis, showed on an x-ray plate an acute enlargement of the heart to the left; the accompanying electrocardiogram showed a decrease in size of all the waves, but especially of the R wave.

In 9 of the 14 rabbits treated with suspensions of streptococci and in all 4 of those receiving diphtheria toxin, diminution in the size of all the waves was seen. This was never found in the controls and only to a very slight extent in the animals treated with adrenalin or

TABLE II.

Rabbit 109 B.

*Received 4 Injections of a Suspension of Streptococcus Isolated after Death from the Heart's Blood of a Patient with Acute Rheumatism. Total Resistance in Circuit for Standardization and for Records, 8,700 Ohms. Deflection Time, 0.02 Seconds throughout Observations.*

Date.	Injection.	Rate.	P-R time.	Size of waves.				Q-R-S time.
				P	R	S	T	
			sec.	mm.	mm.	mm.	mm.	sec.
Mar. 20, 1914	1	170.8	0.07	+1.0	+4.0	-2.5	+3.0	0.025
" " "								
" 23 "		231.6	"	+1.5	+2.5	-3.5	+2.0	0.025
" 24 "		211.4	"	+1.0	+2.0	-2.5	+1.5	0.025
" 25 "		244.8	"	+1.0	+1.0	-2.5	+1.0	0.02
" 28 "		244.5	"	+0.5	+1.0	-1.0	+0.5	0.025
" 30 "		264.5	"	+1.0	+1.5	-2.0	+1.5	0.025
Apr. 2 "	2	230.7	"	+ ?	+1.0	-1.0	+0.5	0.02
" 6 "		253.3	"	+1.0	+3.5	-2.0	+2.5	0.025
" 8 "		228.8	0.06	+1.0	+3.5	-1.5	+1.5	0.02
" 14 "		256.8	"	+1.5	+3.0	-2.5	+2.0	0.025
" 24 "		216.3	0.07	+1.5	+5.0	-2.0	+2.5	0.025
" " "								
May 3 "	3	293.0	"	+1.0	+2.0	-3.0	+1.5	0.03
" 4 "		284.8	"	+1.0	+2.0	-1.0	+1.5	0.025
" 7 "		269.1	0.06	+1.0	+3.0	- ?	+2.0	0.025
" 11 "		238.4	0.07	+1.0	+3.0	-1.0	+2.0	0.025
" 18 "								
" 22 "		278.2	0.06	+1.0	+2.5	-1.0	+1.5	0.025
" 25 "		241.7	0.07	+0.5	+1.0		+1.0	?
" 27 "	4	212.3	"	+0.5	+2.0	-1.0	+1.5	0.025
" 28 "								
June 8 "		218.6	"	+1.0	+3.0	-1.5	+1.0	0.025

spartein and adrenalin, and in them it was a transient effect. Table II, giving the observations on 109 B, illustrates this.

#### DISCUSSION.

Changes in the size and direction of the waves of the electrocardiogram such as those described may depend, as has been pointed out, on changes in the position of the heart in the body, changes in the relative distribution of the muscle mass, and changes in the condition of the muscle and conducting structures. It is possible that conditions of the nervous system may exert an influence from outside the heart. But the influence of the nervous system on the mechanism of the heart beat has been the subject of numerous investigations and changes of this nature have never been demonstrated. The influence of the position of the heart in the body is definitely excluded, as control curves, taken with the animals in different positions before the injections were commenced, showed that in no instance could the changes that resulted be accounted for in this way. In some instances also it was ascertained that alterations in the position of the rabbit could not restore the electrocardiogram to the initial form. The changes in the size and shape of the heart demonstrated by the x-ray examination were always accompanied by changes in the electrocardiogram, and changes in one direction were always accompanied by electrocardiographic changes in a definite direction. It is probable, therefore, that the alterations seen in the roentgenograms were the causes of the electrocardiographic changes described.

The observations extended over seven months in some instances. The records were obtained at all stages between the initial normal forms of the electrocardiograms and the extreme qualitative changes usually associated with right- or left-sided enlargement. These records show that not only the extreme qualitative changes, but also the gradual quantitative decreases and increases in size of the waves of the Q, R, S group seen in the intermediate records are significant of changes in the size and shape of the heart.

Diminution in the size of all the waves was seen in the case of the animals treated with diphtheria toxin. The hearts of these animals showed extreme degenerative changes after death. Acute

degenerative changes have been described by numerous observers after single injections of adrenalin and of spartein and adrenalin. Following the injections, the electrocardiographic records sometimes showed decrease in size of all the waves before the changes associated with the enlargements of the heart manifested themselves and obscured these more general changes. A toxic condition of the muscle and other structures of the heart and degenerative changes in them must alter their functions, and it is not improbable that their electrical activities may be lowered. All the animals injected with streptococci showed joint lesions or altered heart sounds during life, or degenerative changes in the heart muscles after death, so that a similar reason may be present in the nine animals so treated that showed the decrease in the size of all the waves.

The influence of the nervous system and of pathological lesions of the structures themselves on the function of the pace-maker and the auriculoventricular conducting structures has been repeatedly demonstrated. The slowing of the pace-maker and the lengthening of auriculoventricular conduction time and of the duration of the individual waves, seen in the three rabbits treated with fatal doses of diphtheria toxin and in Rabbits 115 B and 119 B treated with streptococci, may have been caused by changes in the nervous mechanism outside the heart. But the intense degenerative changes seen throughout the conducting structures and the heart muscle afford a more probable explanation. Ectopic ventricular contractions can be caused by mechanical or electrical irritation of the heart muscle. The ectopic contractions seen in Rabbit 115 B may well have been due to the foci of degeneration and necrosis seen. Ectopic ventricular contractions were also obtained in Rabbit 118 B although no such foci were found in the sections examined.

A study of the relative weights of the ventricles of the hearts after death showed no changes sufficiently constant to permit of correlation with the electrocardiographic changes.

#### CONCLUSIONS.

1. In rabbits transient and also permanent enlargements of the heart occur as the result of the intravenous injection of adrenalin, spartein and adrenalin, diphtheria toxin, and streptococci.

2. Transient and permanent enlargements of the heart to the left are associated with decrease in the size of the upwardly directed waves of the Q, R, S group in Lead II of the electrocardiograms and increase in the downwardly directed. Transient enlargement to the right is associated with changes in the opposite direction.

3. The stages in the process of enlargement have been observed and are associated with gradual changes in the size of these waves.

4. A diminution in the size of all the waves of the Q, R, S group was observed in degenerative conditions of the heart muscle.

5. Extreme degeneration of the structures of the heart was associated with slowing of the heart's action, lengthening of conduction time (P-Q), and lengthening of the time occupied by the individual waves of the electrocardiogram.

6. Ectopic ventricular contractions were seen in rabbits injected intravenously with suspensions of living streptococci.

#### BIBLIOGRAPHY.

1. Clement, F., *Ztschr. f. Biol.*, 1912, lviii, 110.
2. Erfmann, W., *Ztschr. f. Biol.*, 1913, lxi, 155.
3. Lewis, T., *Lectures on the Heart*, New York, 1915, 3.
4. Einthoven, W., Fahr, G., and de Waart, A., *Arch. f. d. ges. Physiol.*, 1913, cl, 275.
5. Waller, A. D., *Jour. Physiol.*, 1913, xlvi, p. lix.
6. Müller, W., *Die Massenverhältnisse des menschlichen Herzens*, Hamburg and Leipzig, 1883.
7. Fleisher, M. S., and Loeb, L., *Arch. Int. Med.*, 1909, iii, 78.

#### EXPLANATION OF PLATES.

##### PLATE 37.

FIGS. 1 to 5. Rabbit 218 E. Received injections of 0.012 gm. spartein sulphate per kilo and 0.2 cc. of adrenalin hydrochloride 1 : 1,000 (Parke, Davis and Co.) on Dec. 8, 1913, Jan. 2, Jan. 26, Mar. 17, Apr. 6, and Apr. 30, 1914.

FIG. 1. Roentgenogram of heart, Nov. 28, 1913.

FIG. 2. Electrocardiogram, Lead II, Dec. 4, 1913.

FIG. 3. Roentgenogram of heart, May 5, 1914.

FIG. 4. Electrocardiogram, Lead II, May 5, 1914.

FIG. 5. Electrocardiogram, Lead II, Feb. 3, 1914.



## PLATE 38.

FIGS. 6 to 9. Rabbit 211 E. Received injections of 0.012 gm. of spartein sulphate per kilo and 0.2 cc. of adrenalin hydrochloride 1:1,000 (Parke, Davis and Co.) on Dec. 8, 1913, Jan. 5, Jan. 26, Jan. 30, Mar. 17, Apr. 6, and Apr. 30, 1914.

FIG. 6. Roentgenogram of heart, Dec. 4, 1913.

FIG. 7. Electrocardiogram, Lead II, Feb. 3, 1914.

FIG. 8. Roentgenogram of heart, Apr. 15, 1914.

FIG. 9. Electrocardiogram, Lead II, Apr. 16, 1914.

## PLATE 39.

FIGS. 10 to 13. Rabbit 278 E. Received injections of 0.012 gm. spartein sulphate per kilo and 0.2 cc. adrenalin hydrochloride 1:1,000 (Parke, Davis and Co.) on Dec. 8, 1913, Jan. 7, Jan. 26, Mar. 17, Apr. 7, and Apr. 30, 1914.

FIG. 10. Roentgenogram of heart, Dec. 4, 1913.

FIG. 11. Electrocardiogram, Lead II, Dec. 2, 1913.

FIG. 12. Roentgenogram of heart, Apr. 6, 1914.

FIG. 13. Electrocardiogram, Lead II, Apr. 7, 1914 (before injection).

## PLATE 40.

FIGS. 14 and 15. Rabbit 102 B. Received injection of diphtheria toxin, Jan. 29, 1914.

FIG. 14. Electrocardiogram, Lead II, Jan. 29 (before injection).

FIG. 15. Electrocardiogram, Lead II, Feb. 1, 1914.

FIGS. 16 and 17. Rabbit 118 B. Received injections of suspensions of streptococcus isolated from tonsillar abscess, Apr. 22, May 2, May 18, and May 28, 1914.

FIG. 16. Electrocardiogram, Lead II, Apr. 22, 1914 (before injection).

FIG. 17. Electrocardiogram, Lead II, May 8, 1914.

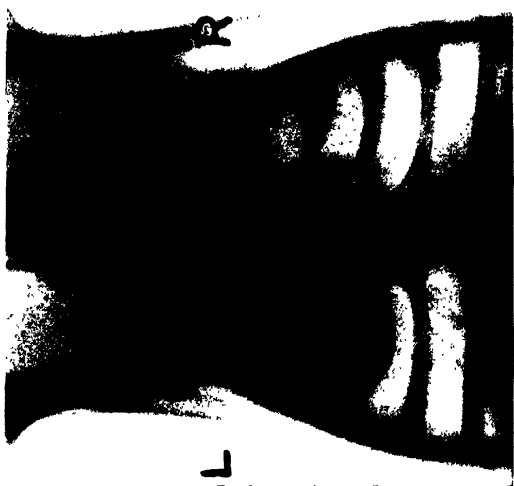


FIG. 3.

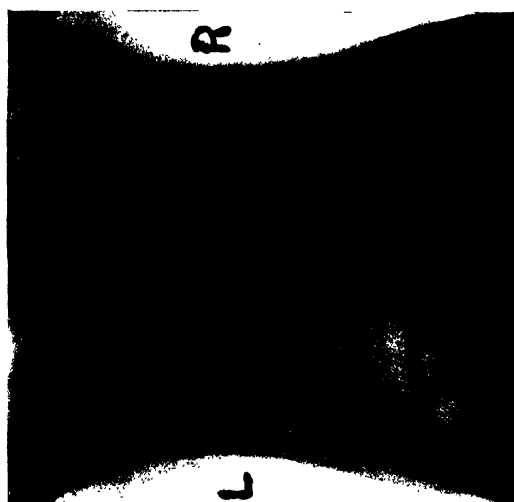


FIG. 1.

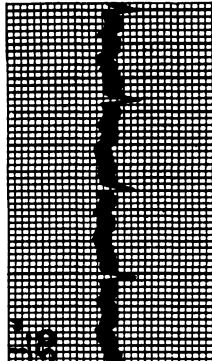


FIG. 5.

(Fraser: Changes in Electrocardiograms.)

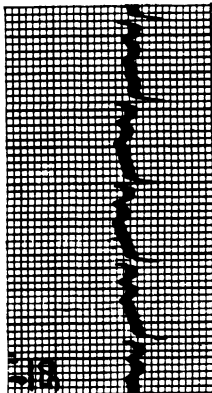


FIG. 4.

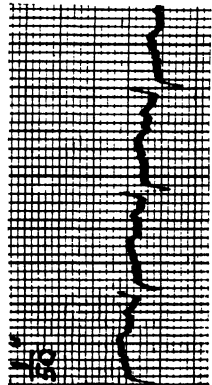


FIG. 2.





FIG. 6.

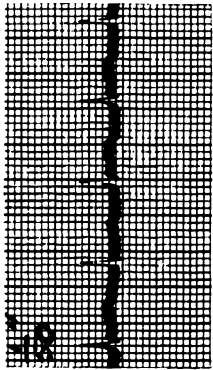


FIG. 8.

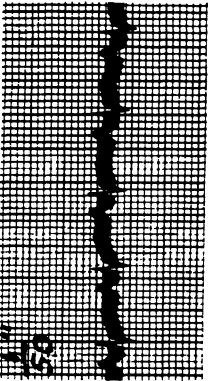


FIG. 7.

FIG. 9.  
(Fraser: Changes in Electrocardiograms.)



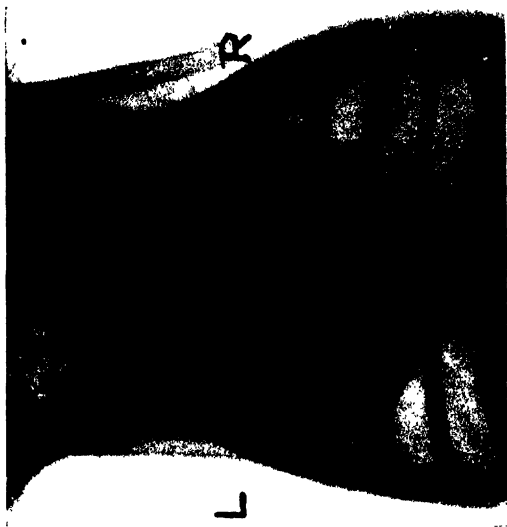


FIG. 10.

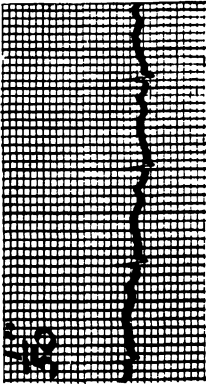


FIG. 11.

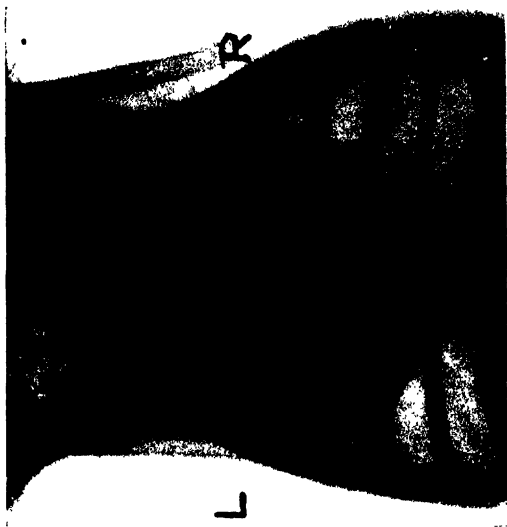


FIG. 12.

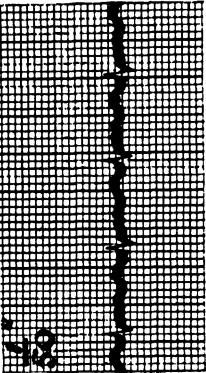


FIG. 13.  
(Fraser: Changes in Electrocardiograms.)



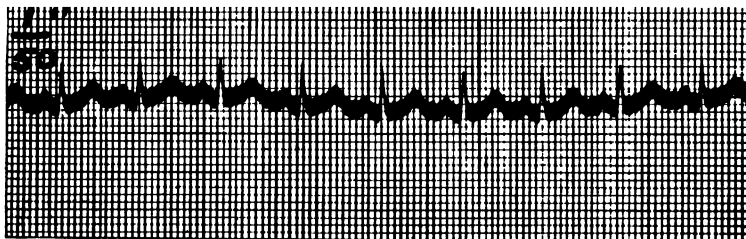


FIG. 14.

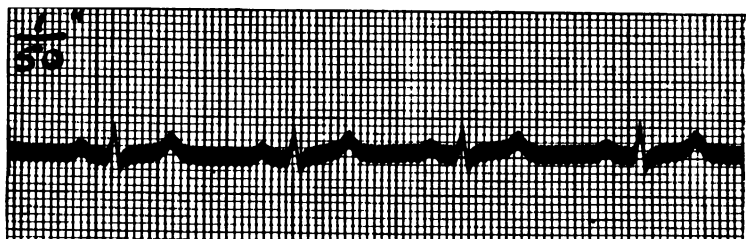


FIG. 15.

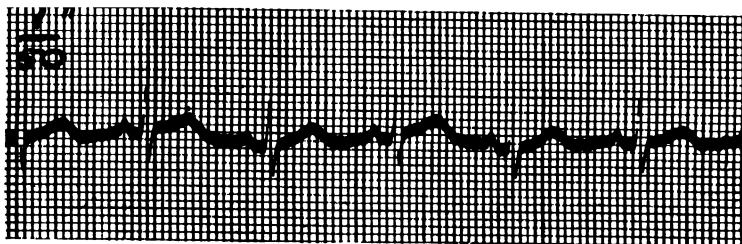


FIG. 16.

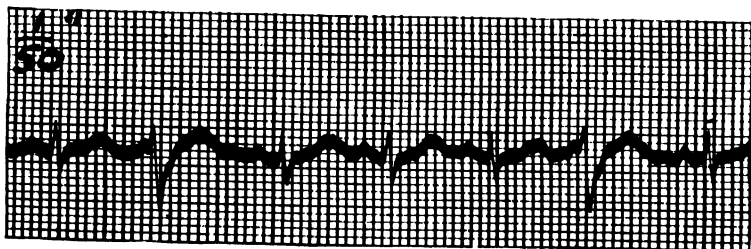


FIG. 17.

(Fraser: Changes in Electrocardiograms.)





## THE NUMERICAL LAWS GOVERNING THE RATE OF EXCRETION OF UREA AND CHLORIDES IN MAN.

### II. THE INFLUENCE OF PATHOLOGICAL CONDITIONS AND OF DRUGS ON EXCRETION.

By FRANKLIN C. McLEAN, M.D.

*(From the Hospital of The Rockefeller Institute for Medical Research,  
New York.)*

(Received for publication, April 17, 1915.)

#### INTRODUCTION.

In a previous communication (1) we have considered the laws governing the rate of excretion of urea and chlorides and their variations in individuals with normal excretion. The present paper deals with the application of these laws to the study of pathological conditions, and to the study of the action of certain drugs on excretion. The aim is for the present not primarily to draw conclusions regarding the diseased conditions themselves, but to offer a means of obtaining more accurate information regarding these conditions than has hitherto been possible. Certain cases which illustrate the nature of the observations made possible by the method have been selected for presentation.

The laws formulated by Ambard for the excretion of urea are already in clinical use in France (2). In the clinic the method has been used almost solely as a measure of renal function, the variations in the values obtained with Ambard's coefficient serving as an indication of disturbed function. Widal, Ambard, and Weill (3) have studied the excretion of chlorides in edematous subjects, and have made some observations on the effect of diuretics. Bauer and Habetin (4) and more recently Bauer and von Nyiri (5), working in Ortner's clinic in Vienna, have published observations, obtained by means of Ambard's laws, on urea and chloride excretion in a considerable number of cases. Inasmuch as they have used an altogether unreliable method for the determination

of urea in the blood, and publish figures as high as 0.779 gm. of urea per liter of serum in individuals with normal excretion, a figure which a person with normal excretion could attain only after ingestion of enormous quantities of urea, our figures can in no way be comparable with theirs.

With the more accurate and rapid methods for urea and chloride determinations now available, requiring only a small amount of blood, it is possible to make frequent and repeated simultaneous observations on urea and chloride excretion in relation to the concentration in the blood in the same individual, and at a minimum of discomfort to him. A wider field is thus opened for investigation. Quantitative studies of human bodily functions could heretofore be carried out only to a limited extent. With the methods here presented, we have a direct measurement of one of the most important excretory functions, elimination of urea through the kidneys, and also a standard for judging the concentration of chlorides in the plasma in its relation to normality. That these methods are more delicate than other methods used for study of disturbed renal function is apparent from the data presented.

### *Methods of Study.*

Simultaneous observations on urea and chloride excretion are made in patients in a manner identical with that described in the preceding paper (1). One-half hour after the patient drinks 150 to 200 cc. of fluid, the bladder is emptied and the subject takes no further fluid or food until a carefully timed period, usually of seventy-two minutes, is ended. The urine excreted during this period is collected, and at the middle of the period about 10 cc. of blood are withdrawn from an arm vein, clotting being prevented by a small amount of powdered potassium oxalate. The choice of a period of seventy-two minutes is merely for the sake of convenience, seventy-two minutes being one-twentieth of twenty-four hours. A one or two hour period may, of course, be used, all calculations in any case being made on a basis of twenty-four hours. In case an error of a few minutes is made in the time of collection of the second specimen, the calculation should be made on the basis of the time actually elapsed between the voiding of the first and second specimens. The amount of urea in the whole blood, and total chlorides, estimated as sodium chloride, in the oxalated plasma after centrifugalization, are determined. Both urea and chlorides are determined in the urine. By substituting the values obtained in the proper formulas the relationship of the rate of excretion of these substances to their concentration in the blood is determined. In the case of urea the rate of excretion under the conditions found is directly measured in terms of the normal, by the use of the following formula:

$$\text{Index of urea excretion} = \frac{8.96 \times \text{Gm. urea per 24 hrs.} \times \sqrt{\text{Gm. urea per liter of urine}}}{\text{Wt. in kilos} \times (\text{Gm. urea per liter of blood})^2}$$

In the normal individual this formula gives a value of about 100 for the index. The theoretical concentration of sodium chloride in the plasma, under the conditions found, is calculated from the following formula:

$$\text{Calculated plasma NaCl} = 5.62 + \frac{\sqrt{\text{Gm. NaCl per 24 hrs.} \times \text{V Gm. NaCl per liter of urine}}}{4.23 \times \text{Wt. in kilos}}$$

For the derivation and significance of these formulas the reader is referred to the foregoing paper. Analyses are carried out by the methods there indicated, and calculations are made with the slide-rule there described.

Observations are made as frequently as desirable. Since they involve no discomfort to the patient, other than the slight inconvenience incident to taking blood, daily observations are usually made when changes in the state of the patient are taking place. The results may be obtained in full within an hour after collection of the specimens, so that information is furnished more rapidly than in other ways. As the observations involve neither additions to the diet nor introduction of foreign substances, they interfere in no way with the dietetic régime or with the progress of the case. The calculations are independent of nitrogen and chloride intake. Gastro-intestinal disturbances, such as vomiting and diarrhea, therefore, do not affect the results, and it is unnecessary to keep the patient on an analyzed diet. Observations can be made at any desired time in almost every case.

Catheterization has been performed only when necessary or advisable in order to avoid danger of losing the specimen, as, for instance, during delirium. It should be practiced in cases in which there is residual urine. Water is given before the seventy-two minute period in order to avoid conditions simulating retention due to dehydration. In the great majority of cases a rate of flow of urine for the period corresponding to 1,000 cc. or more per twenty-four hours is obtained. In practice we make it a rule not to accept results on urea excretion calculated from a urine flow at a rate of less than 500 cc. in twenty-four hours, since results obtained under the condition of a very low rate of urine excretion are apt to be misleading. The chloride formula appears to hold even for very low rates of urinary output.

### *Pathological Conditions Affecting Excretion.*

*Conditions Associated with Retention.*—Retention of nitrogen and salt have long been recognized as occurring in certain forms of cardiac and renal disease. By the use of the present methods new light is thrown on the mechanism of this retention. The salt retention of pneumonia, for example, has been shown to be entirely different in mechanism from the salt retention of chronic nephritis. For our purpose we propose to consider retention only in the sense of its relation to excretion. That is to say, our conception of retention, in the sense just defined, is a relatively high concentration

of substances in the blood in order to induce sufficient excretion. That retention in the tissues themselves occurs is often lost sight of in studies of renal function by methods which require a comparison of intake and output of certain substances. It is manifestly wrong to consider the kidneys responsible for the failure to excrete a certain amount of salt given by mouth, if the salt is taken up by the tissues and the concentration in the plasma remains low. But if the concentration of urea or chlorides in the blood or plasma remains proportionately high, and the rate of excretion proportionately low, it is correct to speak of retention in the sense of a failure to excrete properly. This is the condition in certain types of cardiorenal, or renal disease.

Retention, thus defined, is not an accumulation, but a higher level of substances in the blood, to compensate for increased difficulty in passing through the outlet. It does not mean that the kidney is not able to excrete the normal amount of urea, for the daily excretion, as has repeatedly been shown, may be quite normal. As much may, in fact, be excreted as is absorbed, and the individual be in perfect nitrogen balance (6), although the blood urea or blood nitrogen is much increased. But it does mean that an increased amount in the blood is required in order that increased pressure may be provided to cause the same rate of excretion through diseased kidneys that would be carried on through normal kidneys with a smaller amount in the blood. Actual accumulation may and does occur, under certain conditions. It differs from retention in the above sense, because there is a failure to excrete substances as rapidly as they are absorbed or formed. The picture is different from the purely compensatory phenomenon just described.

The abnormal individual who has retention comes to an equilibrium at a level which is different from that of the normal individual. By comparing his rate of excretion with the standard normal, under the conditions of concentration found in the blood and urine, we obtain a measure of the degree of compensation required to secure the necessary rate of excretion. The necessity for increased pressure may be due to diminished outlet, because a certain amount of the kidney tissue is absent or is not functioning. Thus a per-









son with only one normal kidney, the other being removed, should have an index of urea excretion of approximately 50, 100 representing normality. On the other hand, the kidney cells present may be functionally deficient, though still able to carry out their functions if the pressure under which they work is increased. In either case the index measures the relative functional capacity for the excretion of urea. An index of 100, or 100 per cent, is the standard normal, based on a considerable number of observations on normal individuals. An index of 25 indicates that the individual under observation is excreting urea at 25 per cent of the normal rate under the conditions of concentration of urea found in the blood and urine. An index of 80 is considered as the lower limit of normality, and no normal individual should give a lower figure, providing the fluid intake is sufficient to prevent dehydration.

### *Observations.*

Case I (Text-fig. 1) provided opportunity for making repeated observations during the course of acute nephritis, occurring as a sequel of pneumonia and empyema. Besides fluid intake and output, weight, concentration of albumin in the urine, systolic and diastolic blood pressure, P-R time measured electrocardiographically, pulse rate, and temperature, Text-fig. 1 illustrates the findings as regards urea and chloride excretion, with occasional observations on the rate of excretion of phenolsulphonephthalein. The concentration of urea in the blood and the rate of excretion are shown, together with the index of urea excretion, calculated as above indicated. It will be seen that during the first two months of the disease, though the level of the blood urea fluctuated considerably, due to changes in the nitrogen intake, the index remained remarkably constant at 6 to 8. The first fall in blood urea followed an initial restriction of diet, and was accompanied by a proportionate decrease in the rate of excretion. As the nitrogen intake increased, the blood urea rose steadily, reaching a level of 1.3 grams per liter and maintaining this level for some time. The rate of excretion at the same time increased proportionately, so that the index remained constant. When functional improvement did occur it was accompanied by a rapid

fall in the blood urea, and an increase in the index toward normal. The rate of excretion during and following this fall remained practically the same as when the high concentration of urea in the blood was found. The maintenance of a constant relationship of rate of excretion to the concentration in the blood, at 6 to 8 per cent of normal, under fluctuating conditions of nitrogen intake, indicates that the process of excretion was being carried out under laws just as definite as those governing the normal excretion. During this period the actual rate of excretion maintained was as high as later in the disease when the conditions were more nearly normal. During recovery the only changes were the fall in concentration of urea in the blood and the rise in the value of the index. One must conclude, therefore, that the high concentration of urea in the blood was maintained in order to compensate for the difficulty in passing through a damaged outlet in the form of kidneys which were functioning at a rate only 6 to 8 per cent of normal. When functional conditions improved, it was no longer necessary to maintain the concentration of urea in the blood at so high a level, and it promptly fell to within normal limits. The relation of the rate of phthalein elimination to the urea index is shown in this case. Both increased at the same time, the increase being parallel. The failure of the index to reach the normal figure for some time, in spite of the fact that the blood urea was within normal limits, shows that the blood urea figure alone was not sufficient in this case to indicate the functional disturbance which still existed.

Text-fig. 1 also shows the rate of chloride excretion, maintained at a low level by a salt-poor diet; the theoretical concentration of sodium chloride in the plasma, calculated from the rate of excretion from the formula given above; and the concentration of sodium chloride found in the plasma by analysis. A marked discrepancy is seen between the actual and calculated concentrations of sodium chloride in the plasma, the former being always higher, and reaching at one time a level of over 7 grams per liter. A return of the plasma chlorides toward the theoretical concentration was much slower than the fall in blood urea, and confirms previous experience that chloride function may be disturbed for some time after ap-

parent recovery. The case also shows the independence of the urea and chloride functions, since the urea function improved so rapidly some time before any change in the chloride relations was visible.

Drug experiments in this case were entirely negative. Edema was present at the start, but disappeared rapidly under free catharsis and sweat baths. The course of the edema is indicated by the weight chart. The later increase in weight was a healthy increase and was not accompanied by any edema.

Case II (Table I) is one of acute nephritis following pneumonia, similar to, but much milder than, the previous one. The urea index remained fairly constant at about 75 for a time, and then rose to a point considerably higher than normal. The chloride excretion followed a similar course, the concentration of sodium chloride found in the plasma being higher than the theoretical concentration during the first stage, and lower during the stage in which the urea index was high. This represents a stage of vascular irritability, which has been described qualitatively by Schlayer (7) and others, but this method offers a means of measuring such changes quantitatively. Recovery in this case is accompanied by a return of both urea and chloride functions toward normal.

TABLE I.

*Case II.*

Date (1915).	Weight.	Urine per 24 hrs.	Urea.				Sodium chloride.					Urine findings.		
			Gm. per liter of blood Ur.	Gm. per liter of urine C.	Gm. per 24 hrs. D.	In- dex of ex- cretion I.	Gm. per liter of urine C.	Gm. per 24 hrs. D.	Gm. per liter of plasma.			Blood.	Albumin.	Casts.
									Cal- culated.	Ac- tual.	Dif- ference.			
	<i>kilos</i>	<i>cc.</i>												
Mar. 9	51.0	1,100	0.562	26.2	28.8	83	0.9	0.99	5.69	5.60	-.09	++	H.T.*	+++
" 10	51.0	2,070	0.577	20.9	43.3	104	0.7	1.5	5.70	5.65	-.05			
" 11	51.0	1,195	0.627	27.0	32.3	75	0.6	0.7	5.67	5.81	+.14	o	F.T.	+
" 12	51.0	1,200	0.557	23.3	28.0	77	2.0	2.4	5.75	5.97	+.22			
" 13	51.0	1,600	0.506	16.4	26.3	71	5.2	8.3	5.92	6.15	+.23	o	V.F.T.	+
" 18	51.0	2,034	0.547	15.35	31.2	72	9.3	18.9	6.14	6.17	+.03		V.F.T.	Few.
" 22	53.0	1,500	0.557	17.1	26.6	58	8.3	12.5	6.02	6.08	+.06		V.F.T.	"
" 26	53.8	1,440	0.457	16.3	23.4	75	10.9	15.7	6.10	6.06	-.04		V.F.T.	++
Apr. 2	56.0	6,000	0.321	9.0	54.0	250	7.3	43.8	6.33	6.03	-.30		V.F.T.	++
" 9	56.2	6,300	0.308	10.0	63.0	330	9.8	61.8	6.52	6.21	-.31		V.F.T.	+
May 30	57.0	1,500	0.250	15.9	23.8	240	13.5	20.2	6.17	6.19	+.02		V.F.T.	Few.

\* In Table I, H.T. signifies heavy trace; F.T., faint trace; and V.F.T., very faint trace.

*Case II.*—(Table I.) D. C., male, aged 16. Diagnosis, lobar pneumonia, acute nephritis. Admitted Mar. 8, 1915, on 6th day of pneumonia. Temperature 105.5° F. Consolidation posteriorly in right lower lobe. Blood pressure 112 systolic, 60 diastolic. Mar. 10. Crisis, followed by rapid disappearance of physical signs of consolidation. No edema at any time; no further abnormal physical signs, except for slight pallor. Mar. 26. Discharged from hospital, feeling well except for slight weakness. Blood pressure 102 systolic, 60 diastolic. May 30. Patient has been working for a month; feels perfectly well. Blood pressure 108 systolic, 70 diastolic.

In Case III (Table II) enormous pressure was required in order to induce the necessary rate of excretion of urea and sodium chloride. The close agreement of the urea index with the rate of phthalein excretion is also seen in this case.

Attention has been called by Widal and Javal (8), and more recently by Folin, Denis, and Seymour (9), and by Frothingham and Smillie (10) to the reduction in blood urea or blood nitrogen brought about by giving a low protein diet. They state that in cases with high blood nitrogen, such as in this case (Case III, Table II), no marked reduction occurs on a nitrogen-poor diet. That any considerable reduction is difficult in a case such as this may be shown mathematically. Were this patient to attain a blood urea figure of 0.42 grams per liter, that is to say, one within normal limits, the total urea excretion per twenty-four hours in 1 liter of urine would be 1 gram, the index remaining at 1.2. The formula would then read 
$$\text{Index} = \frac{8.96 \times \sqrt{1}}{42.1 \times (0.420)^2} = 1.2.$$
 As the concentration of urea is practically the same in all the tissues as in the blood, before the concentration of over 2 grams per liter could be reduced to 0.42 grams per liter this patient would have to excrete nearly 80 grams of urea already in the body. As the rate of excretion would diminish in proportion to the square of any diminution in the concentration in the blood, it would require an infinite time for the blood urea figure to reach the low level, even if the diet could be kept so that only 1 gram of urea were formed daily. The practical impossibility of reaching such a result is obvious. The comparative ease with which a patient with an index of urea excretion more nearly normal would reach a blood urea figure within normal limits when on a low nitrogen diet may easily be seen.

*Case III.*—(Table II.) J. O. S., male, aged 13. Diagnosis, chronic nephritis, mitral stenosis. Admitted Jan. 19, 1915, with generalized edema, dyspnea, frequent nausea, and vomiting. Blood pressure 185 systolic, 135 diastolic. Urine clear, straw colored, specific gravity 1008, acid, no sugar. Albumin, 6.5 gm. per liter (Esbach). Many large granular casts, hyalin and epithelial casts, epithelial cells, and leucocytes. No red cells. Eye grounds normal. Jan. 20. Phenol-sulphonephthalein, 2 per cent excreted in 2 hours. Jan. 26. Left hospital unimproved. Died one month later.

TABLE II.

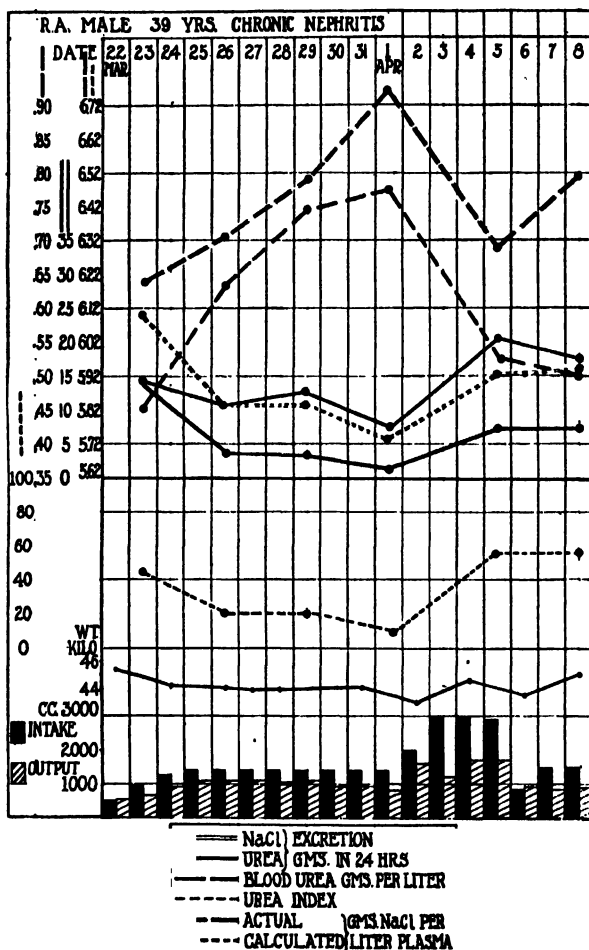
*Case III.*

Date (1915).	Weight.	Urine per 24 hrs.	Urea.				Sodium chloride.				
			Gm. per liter of blood Ur.	Gm. per liter of urine C.	Gm. per 24 hrs. D.	Index of excre- tion I.	Gm. per liter of urine C.	Gm. per 24 hrs. D.	Gm. per liter of plasma.		
	kilos	cc.							Calcu- lated.	Actual.	Differ- ence.
Jan. 20...	42.1	889	2.147	8.8	7.82	1.0	1.5	1.33	5.71	6.55	+ .84
" 22...	42.1	960	2.203	9.37	9.0	1.2	2.0	1.92	5.75	6.59	+ .84

Case IV (Text-fig. 2) illustrates an actual accumulation of urea and chlorides, due to restriction of fluids while on a full diet. That the power of excreting substances in high concentration in the urine is diminished in certain forms of nephritis is well recognized. It is quite apparent that a fluid output insufficient to carry off the waste products formed will lead to actual accumulation of these products in the body. This condition is manifested, in the case presented, by the increasing urea and chloride content of the blood and the diminishing index of urea excretion. When the fluid intake is increased, urea, chlorides, and index return rapidly to their former state. This case also illustrates the necessity of careful control of the fluid intake in any experiments on nitrogen or chloride balance. That a washing out of urea and chlorides occurs in both normal and pathological individuals with a high fluid output is recognized and expressed in the formulas. Any experiment aiming to determine the balance between nitrogen or chloride intake and output, which does not take the fluid output into account, is apt to be misleading.

Conditions similar to those occasioned by disturbance of function due to renal disease may also be found in heart failure, with passive

congestion of the kidneys. In case the circulation alone is at fault the conditions return to normal when the congestion is relieved by digitalis or otherwise.



TEXT-FIG. 2. Case IV. Chronic nephritis. Blood pressure 185 systolic, 130 diastolic. Urine clear, amber, specific gravity 1023, acid, no sugar, albumin 2 gm. per liter (Esbach). Numerous hyalin, few granular casts. Red blood cells and white blood cells. Mar. 23 to Apr. 2. Fluids restricted, food and salt *ad libitum*. After Apr. 2 fluids increased and food and salt restricted.

Case V (Table III) illustrates the findings in a typical case of heart failure with passive congestion of the kidneys, which was

restored to a functionally normal state by digitalis. During the condition of passive congestion, as evidenced by cyanosis, edema, and albuminuria, the blood urea was high, the urea index low, and the plasma chlorides high in relation to the rate of excretion. Under the action of digitalis the blood urea fell, the index rose to normal, and the plasma chlorides fell until the concentration agreed with the theoretical concentration as calculated from the rate of excretion. The urine became albumin-free and no casts were to be found, indicating complete relief from the congestion which had caused disturbed function.

*Case V.*—(Table III.) M. H., male, aged 53. Diagnosis, aortic and mitral insufficiency, cardiac failure, passive congestion of kidneys. Admitted Jan. 2, 1915, with dyspnea, cyanosis, edema of extremities. Blood pressure 143 systolic, 110 diastolic. Urine dark amber, cloudy, specific gravity 1027, acid, albumin ++, numerous hyalin and granular casts. Jan. 8. Condition unchanged by rest in bed; digitalis therapy started (digipuratum 0.1 gm., 4 times daily). Jan. 12. Patient much more comfortable, edema disappearing, fluid output in excess of intake. Jan. 17. Digitalis discontinued; total 2.9 gm. Jan. 19. No edema, or dyspnea. Urine albumin-free, no casts. Blood pressure 120 systolic, 75 diastolic. Patient continued in good condition up to time of discharge on Apr. 11.

TABLE III.

*Case V.*

Date (1915).	Weight.	Urine per 24 hrs.	Urea.				Sodium chloride.					Urine albumin, gm. per liter (Esbach).	Medication.
			Gm. per liter of blood Ur.	Gm. per liter of urine C.	Gm. per 24 hrs. D.	Index of excretion I.	Gm. per liter of urine C.	Gm. per 24 hrs. D.	Gm. per liter of plasma.				
									Calcu- lated.	Actual.	Differ- ence.		
Jan. 4..	<i>kilos</i> 83.1	<i>cc.</i> 500	0.567	27.3	13.65	24	3.3	1.65	5.71	5.84	+ .13	1.5	Digitalis. "
" 8..	77.0	420	0.597	30.8	12.94	25	3.35	1.41	5.70	6.15	+ .45	0.5	
" 12..	74.0	3,020	0.351	11.84	35.7	130	8.9	26.9	6.07	6.12	+ .05	0.25	
" 15..	73.0	1,190	0.356	20.7	24.7	100	6.9	8.21	5.88	5.91	+ .03	0	
" 27..	71.0	1,620	0.328	15.7	25.5	110	7.7	12.5	5.96	5.85	- .11	0	
Feb. 16..	73.2	1,000	0.286	16.42	16.42	100	12.4	12.4	5.99	5.96	- .03	0	

Case VI (Table IV) is a case of heart failure in which actual accumulation of urea and chlorides in the blood occurred. This is evidenced by the very rapid rise in the concentration of both urea and chlorides in the blood, without any increased rate of excretion. This picture, with its rapidly diminishing urea index, is quite dif-

ferent from that presented by Cases I and III, in which a persistently high blood urea was accompanied by a constant value for the index.

*Case VI.*—(Table IV.) A. W., female, aged 13. Diagnosis, mitral insufficiency, auricular fibrillation, cardiac failure, death. First admitted Apr. 17, 1913. Last admission, Dec. 3, 1914, with edema, dyspnea, and cyanosis. Urine amber, cloudy, specific gravity 1030, acid, albumin + + +, few granular casts. Blood pressure 120. Dec. 12. Phenolsulphonephthalein, 68 per cent excreted in 2 hours. Patient's condition failed to improve materially under digitalis, and about Dec. 28 she became decidedly worse, gradually stuporous, and more cyanotic. Edema rapidly disappeared during last few days, and blood contained a very high proportion of cells and a low percentage of plasma. Jan. 10. Died, after having been unconscious two days. Autopsy revealed enormously enlarged and dilated heart, with lesion of mitral valve. The kidneys were those of passive congestion.

TABLE IV.

*Case VI.*

Date (1914).	Weight.	Urine per 24 hrs.	Urea.				Sodium chloride.					Urine albu- min, gm. per liter (Es- bach).
			Gm. per liter of blood Ur.	Gm. per liter of urine C.	Gm. per 24 hrs. D.	Index of ex- cre- tion I.	Gm. per liter of urine C.	Gm. per 24 hrs. D.	Gm. per liter of plasma.			
									Calcu- lated.	Ac- tual.	Differ- ence.	
	<i>kilos</i>	<i>cc.</i>										
Dec. 12 ...	37.9	680	0.460	23.75	16.15	88	7.0	4.75	5.90	6.15	+ .25	
" 21 ...	38.8	540	0.432	22.05	11.9	70	3.3	1.8	5.76	5.95	+ .19	3.0
(1915)												
Jan. 5 ..	37.0	1,850	0.837	11.13	20.6	24	2.4	4.44	5.83	7.24	+1.41	1.5
" 7 ...	32.0	1,000	1.38	12.25	12.25	6	3.7	3.7	5.85	7.85	+2.00	2.5
" 8 ...	30.0	500	2.18	17.85	8.92	2	1.1	0.55	5.69	8.44	+2.75	0.5
" 9 ...			2.70							8.24		
" 10 ...			3.36									0.1

We may summarize the findings in cases with retention as follows. In individuals with defective elimination of urea or chlorides, an increased concentration of these substances in the blood is required to compensate for the faulty excretion. Under the conditions of increased concentration in the blood, the actual rate of excretion and the balance between intake and output may be quite as good as in a perfectly normal individual. The increased concentration in the blood is not to be regarded as an accumulation unless the concentration in the blood is increasing and is associated with a diminished elimination. In any given case the index of urea ex-



cretion measures, in its relation to normality, the rate of excretion under the conditions found. Increased chloride concentration in the plasma is shown by comparing the amount actually found with the theoretical concentration, calculated from the rate of excretion. The concentration in the blood and the rate of excretion of urea and chlorides depend not only on the nitrogen and chloride intake, but on the fluid intake and output. Deficient elimination due to passive congestion may be restored to normal if the circulatory failure is relieved.

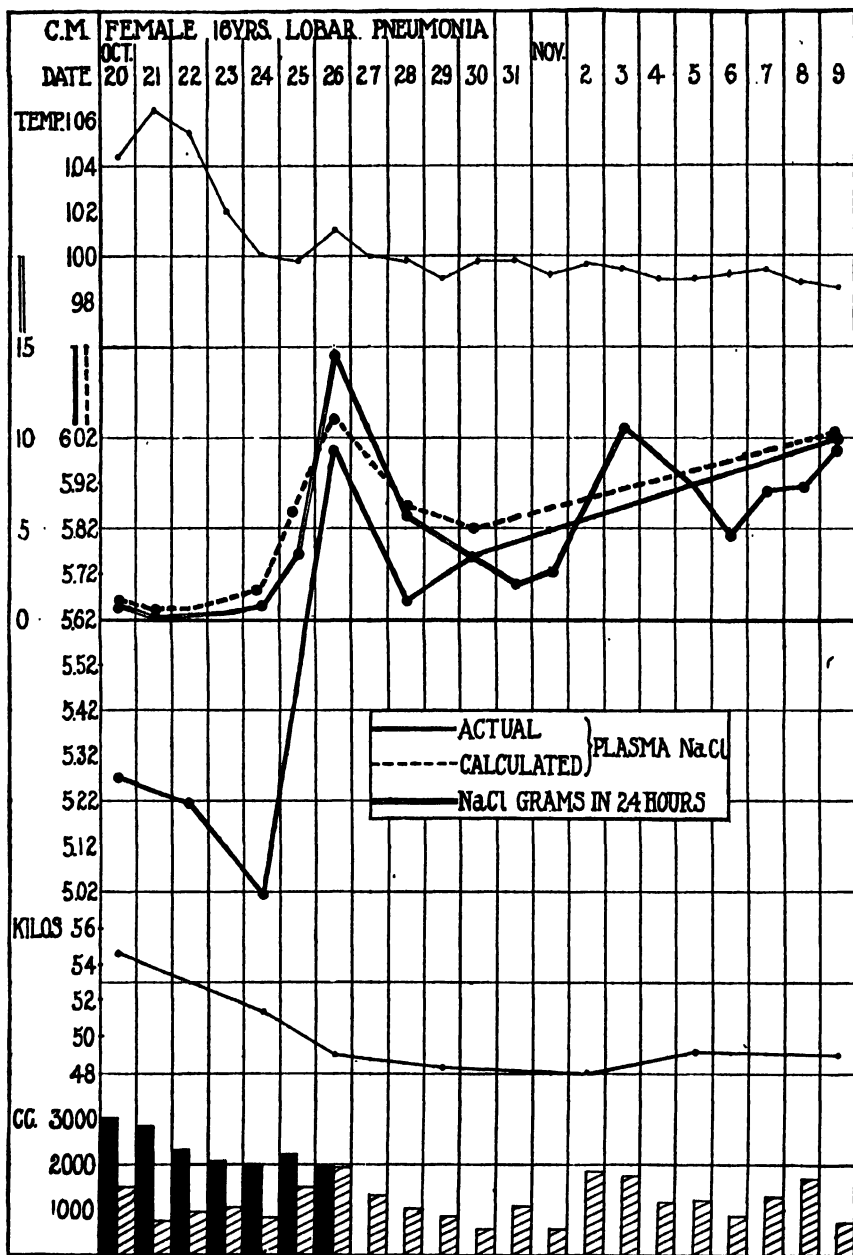
### *The Chlorides in Pneumonia.*

That the chloride retention in pneumonia is associated with a diminished chloride content in the blood has been shown by Peabody (11) and others. Peabody has also shown that the concentration of chlorides in the blood increases at the time excretion begins. This is directly opposed to the condition in nephritis, where chloride retention is usually associated with a high chloride content in the plasma, and suggests at once that the chloride retention occurring in pneumonia and other fevers is not due to a failure on the part of the kidneys to excrete chlorides, as some writers still hold (12).

We have examined the chloride content of the plasma and its relation to the rate of chloride excretion in 60 observations in 13 cases of lobar pneumonia, and have found that the failure to excrete chloride during the acute stage of the disease is almost always associated with a concentration of sodium chloride in the plasma below 5.62 grams per liter (the normal threshold).<sup>1</sup> One must believe that this is the cause of failure to excrete chlorides, since excretion begins with a rise in the chloride content of the plasma.

Case VII (Text-fig. 3) illustrates the findings regarding chloride excretion in a typical case of uncomplicated pneumonia. During the acute stage of the disease, when only a very small amount of chlorides appeared in the urine, the plasma content of sodium chloride was considerably below 5.62 grams per liter. At the crisis the plasma chlorides rose abruptly, and excretion began and continued

<sup>1</sup> Similar findings have been reported by J. Snapper (*Deutsch. Arch. f. klin. Med.*, 1913, cxi, 429) in 5 observations during the chloride retention of pneumonia.



TEXT-FIG. 3. Case VII. Uncomplicated lobar pneumonia, with recovery.

in proportion to the concentration in the plasma. That the low rate of excretion of chlorides in pneumonia is not due to chloride starvation has been repeatedly shown (11). That the low chloride content of the plasma is not due to low intake is shown by the rapid rise in Case VII at the time of crisis, without any increase in the chloride intake. While the finding of a low concentration in the plasma shows that the kidneys are not responsible for salt retention in pneumonia, it does not explain the mechanism of the retention, which requires further investigation.

### *The Chloride Threshold in Fever.*

We have calculated the threshold in cases with fever from the formula used in normal individuals (1):

$$\text{Threshold} = \text{Plasma NaCl} - \sqrt{\frac{\text{Gm. NaCl per 24 hrs.} \times \text{Gm. NaCl per liter of urine}}{4.23 \times \text{Wt. in kilos}}}$$

For the most part the threshold, as calculated by this formula, is actually lowered during the chloride retention of pneumonia, the average threshold in our cases being 5.42 as opposed to the normal threshold of 5.62 grams of sodium chloride per liter of plasma. That a similar condition occurs in other fevers is shown by Case VIII (Table V).

*Case VIII.*—(Table V.) B. G., female, aged 20. Diagnosis, acute rheumatic fever. Admitted Nov. 20, 1914. Nov. 27. Pain and swelling in joints. Temperature 103° F. Urine amber, cloudy, specific gravity 1019, alkaline, faint trace of albumin, no casts, few leucocytes.

TABLE V.  
*Case VIII.*

Date (1914).	Weight.	Urine per 24 hrs.	Sodium chloride.					Thresh- old.
			Gm. per liter of urine C.	Gm. per 24 hrs. D.	Gm. per liter of plasma.			
					Calcu- lated.	Actual.	Differ- ence.	
Nov. 27. ....	<i>kilos</i> 60.0	<i>cc.</i> 1,285	2.8	5.1	5.80	5.54	— .26	5.36

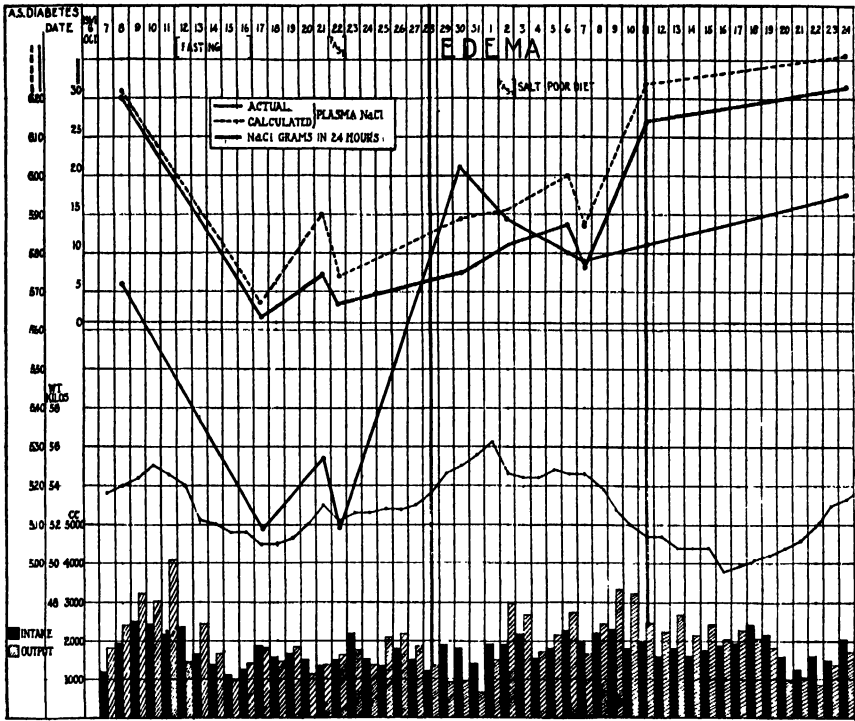
In some instances the threshold was never materially lowered, but in no case was it appreciably raised during the acute stage of the dis-

ease. In one case of chronic interstitial nephritis, dying of pneumonia, with high blood urea, the chloride threshold was considerably lowered. In one case it was raised temporarily during convalescence. As a rule, a lowered threshold returns to normal at the time excretion begins. One case showed a threshold still lowered at the time of discharge from the hospital, after complete recovery.

The findings regarding chlorides in pneumonias and other fevers may be summarized as follows: Chloride retention in pneumonia is associated with a lowered concentration of chlorides in the plasma, and failure of excretion is apparently due to this cause. The threshold is often considerably lowered during fever, and usually returns to normal after the temperature becomes normal. A raised threshold has not been observed during acute fever.

#### *The Chlorides in Diabetes (13).*

In 78 observations in 28 cases of diabetes mellitus, mainly severe cases, we have found in a majority a lowering of the chloride threshold. Such a condition is shown in Case IX (Text-fig. 4). In this case, under varying conditions of diet, excretion of sugar, and concentration of sugar in the blood, the plasma chlorides remained proportionately very low, corresponding to a threshold of about 5.12 instead of 5.62 grams of sodium chloride per liter of plasma. Only when the patient developed an edema, such as that to which diabetics are subject, did the plasma chlorides show a relative increase, returning to their previous relationship to the rate of excretion with the disappearance of the edema.



TEXT-FIG. 4. Case IX. Oct. 7. Urine clear, amber; sugar, 75 gm. in twenty-four hours; albumin 0; casts 0. Ferric chloride reaction heavy. Fasted Oct. 12 to 18, with whiskey. No alkalis. Oct. 18. Sugar 0; albumin 0. Ferric chloride reaction very faint. Urine remained sugar-free, with the exception of occasional traces when the increase in diet was not tolerated. Nov. 1. Well marked edema of both feet and ankles, which remained until Nov. 11. Urine, albumin 0, no casts. Had similar edema at subsequent times.

Case X (Table VI) illustrates the findings in a typical case with constantly lowered threshold. In this case the actual concentration of sodium chloride in the plasma is almost invariably below 5.62 grams per liter, the normal threshold.

The average of the threshold calculated by the above formula in 78 observations in diabetics is 5.34, in contradistinction to the average of  $5.61 \pm$  grams in all normal individuals. Up to the present time we have not succeeded in associating this change in chloride threshold and excretion with any other peculiarities of individual cases of diabetes. It seems to bear no relation to the amount of

sugar excreted nor to acidosis. It seems to occur most commonly in the more severe cases, but has also been seen in milder cases.

Case X.—(Table VI.) J. U., male, aged 44. Diagnosis, diabetes mellitus.

TABLE VI.

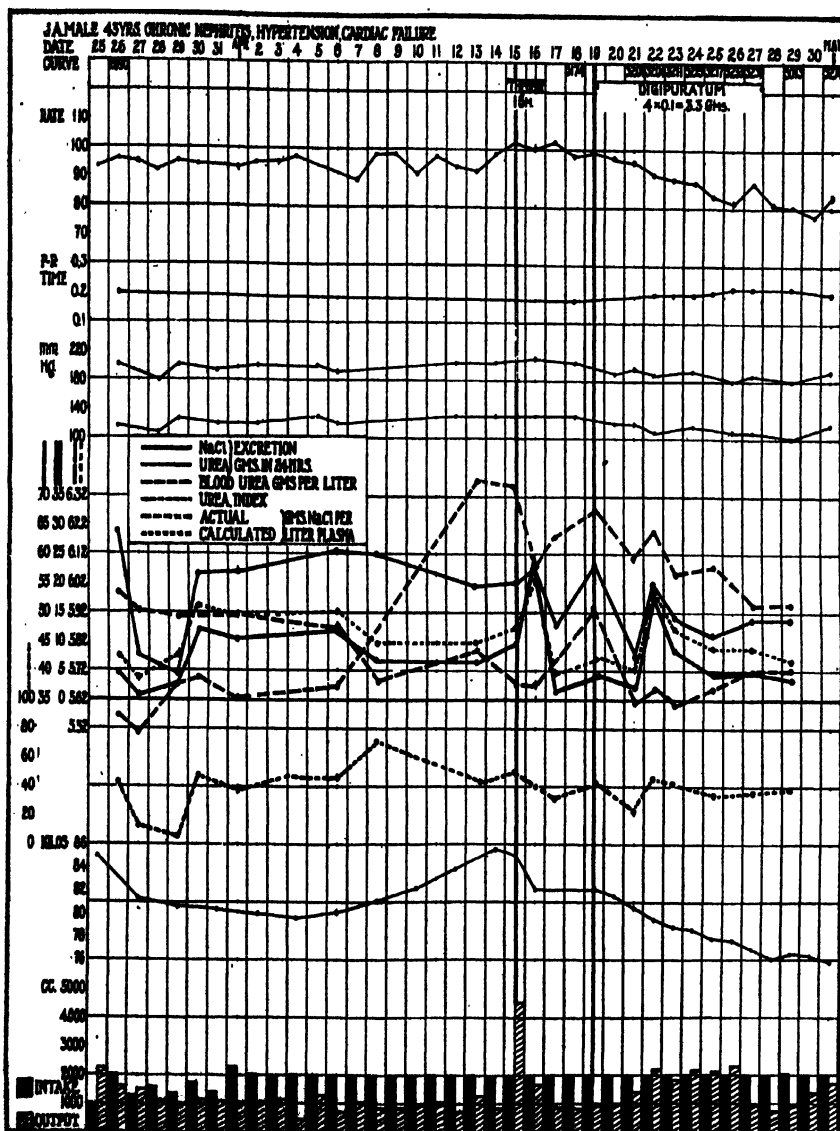
Case X.

Date (1914).	Weight.	Urine per 24 hrs.	Sodium chloride.						Sugar in 24 hrs.	Ferric chloride reaction.	Diet per 24 hrs.	
			Gm. per liter of urine C.	Gm. per 24 hrs. D.	Gm. per liter of plasma.			Threshold.			Carbohy- drate.	Total calories.
					Calcu- lated.	Actual.	Differ- ence.					
Dec. 3..	kilos 45.0	cc. 860	1.9	1.63	5.73	5.00	-.73	4.89	gm. 2.73	+++	Fasting with alcohol 3d day.	
" 7.. (1915)	45.0	655	2.4	1.6	5.73	5.08	-.65	4.97	0	+	0	637
Jan. 6..	41.3	1,850	11.13	20.6	6.23	5.35	-.88	4.74	0	+	0	466
May 4..	42.6	950	10.1	9.6	6.03	5.87	-.16	5.46	0	+	40.1	343.5
" 7..	44.0	800	9.8	7.85	5.98	5.22	-.76	4.86	0	+	70	600.5
" 12..	44.0	1,320	9.2	12.1	6.06	5.48	-.58	5.04	Trace	+	10	553
" 13..	43.8	1,590	9.4	14.9	6.12	5.60	-.52	5.10	0	+	20	929
" 18..	44.0	690	10.0	6.9	5.96	5.40	-.56	5.12	Trace	+	15.1	1,526.9
" 22..	44.0	700	14.3	10.0	6.07	5.70	-.37	5.25	0	+	0	1,427
June 2..	43.0	800	9.5	7.6	5.98	5.42	-.56	5.06	0	+	0	1,962

### *The Influence of Drugs on Excretion of Urea and Chlorides.*

Study of the effects of drugs has so far been confined mainly to the action of digitalis. We have found, in the majority of cases examined, a lowering of the chloride threshold as the result of giving digitalis, both in individuals with normal excretion and in cases with passive congestion. The threshold may be brought merely to normal, as in Case V (Table III), or it may be brought temporarily far below normal, as in Case XII (Table VII).

Case XII.—(Table VII.) G. M., male, aged 50. Diagnosis, cardiac hypertrophy, auricular fibrillation. Patient has been under observation since May 19, 1914. Rate remains slow and patient feels well when taking digitalis, but rate becomes rapid and dyspnea and edema appear when digitalis is omitted. Oct. 5, 1914. Patient has had no digitalis since Sept. 27. Pulse rate gradually increasing. Urine clear, amber, specific gravity 1025, neutral, albumin 0.25 gm. per liter (Esbach), no casts found. Oct. 16. Marked dyspnea, face puffy, heart rate rapid. Output of fluid has diminished and weight increased. Digitalis started. Oct. 23. Fluid output increased and patient much better. No edema.



TEXT-Fig. 5. Case XI. Chronic nephritis, hypertension, and cardiac failure. Urine clear, amber, specific gravity 1007, acid, albumin heavy trace, numerous hyalin and granular casts. Admitted under influence of infusion of digitalis. Later treated with theocin and with digipuraturn.





Nov. 2. Patient feels well; rate remains slow. Two similar series of observations have since been made on the same patient.

TABLE VII.

## Case XII.

Date (1914).	Weight.	Urine per 24 hrs.	Urea.				Sodium chloride.					Phthalein excreted.		
			Gm. per liter of blood Ur.	Gm. per liter of urine C.	Gm. per 24 hrs. D.	Index of excretion I.	Gm. per liter of urine C.	Gm. per 24 hrs. D.	Gm. per liter of plasma.			1st hr.	2d hr.	Total.
									Calcu- lated.	Actual.	Differ- ence.			
	<i>kilos</i>	<i>cc.</i>										<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Oct. 5..	71.0	760	0.400	18.2	13.85	53	13.0	9.8	5.96	6.11	+ .15	38		68
" 16..	73.0	685	0.500	26.82	18.37	47	5.1	3.5	5.78	5.97	+ .19		30	
" 23..	72.6	1,600	0.352	13.15	21.0	76	10.0	16.0	6.03	5.51	- .52	Medication.		
" 26	72.0	1,680	0.358	13.68	22.9	82	12.3	20.7	6.11	6.11	0	Digitalis.		
Nov. 2..	72.4	1,580	0.396	14.02	22.3	66	12.1	19.1	6.09	6.10	+ .01			

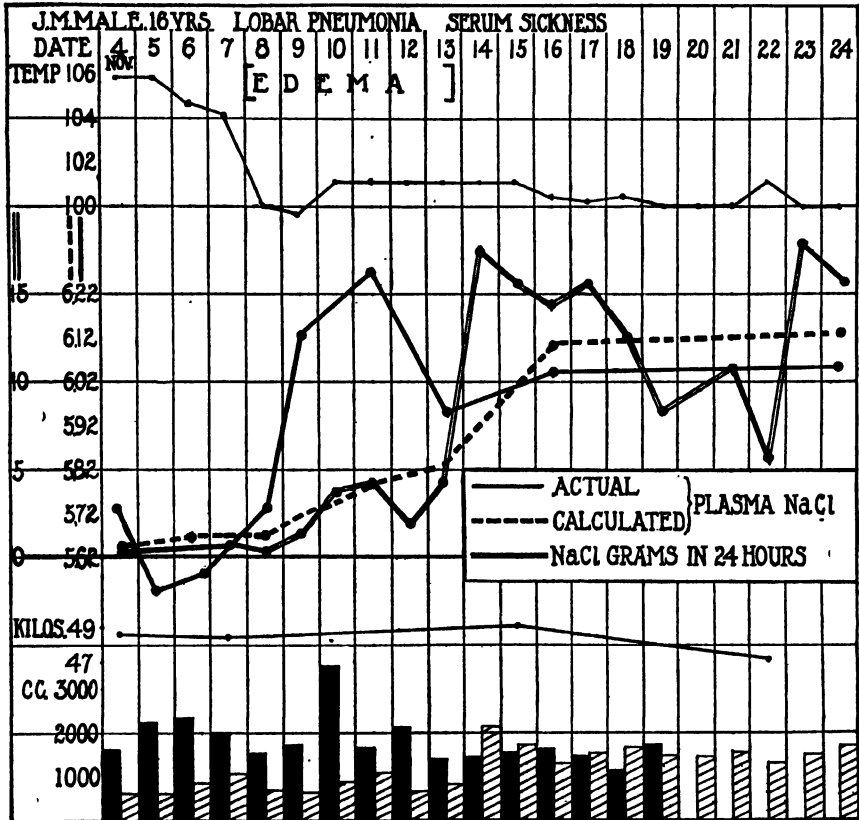
Urea excretion is increased after passive congestion is relieved by digitalis, but digitalis seems to have no specific direct effect on urea excretion. Case XI (Text-fig. 5) shows the effect of both digitalis and theocin, as separate observations on the same individual. Both the chloride and urea findings are illustrated, as in Text-fig. 1. Except for the temporary fall in the urea index at first, caused by insufficient fluid, the index remains practically unchanged, and shows no response to drug therapy. The constantly low index is apparently due to a disturbance in function due to chronic nephritis, with hypertension. When admitted, the patient was under the influence of large doses of infusion of digitalis, and the plasma chlorides were very low, the concentration being below the normal threshold. As the influence of digitalis disappeared, the plasma chlorides rose rapidly, edema appearing and increasing with the increase in plasma chlorides. Diuresis with theocin increased the chloride excretion and brought down the plasma chlorides temporarily to the theoretical concentration. Conditions rapidly returned to the previous state, until digitalis was again administered. At this time the plasma chlorides did not fall to the previous low level, though they responded to digitalis therapy with a decrease in concentration, approaching but not reaching the theoretical concentration.

Edema, however, disappeared and the patient gave other evidences of a favorable reaction to digitalis. Since this time the patient has been out of the hospital, and has returned with edema. At this later time, not shown in Text-fig. 5, digitalis caused the excretion of 11,000 cc. of urine in forty-eight hours, and the plasma sodium chloride fell to 5.37 grams per liter and remained low for some time. The index of urea excretion was exactly the same during this enormous diuresis as on the preceding day, when the fluid output was only 900 cc. The length of time for which the chloride threshold remains low after digitalis varies in different cases, but it may remain low for ten days or longer after digitalis is discontinued.

Digitalis and diuretin were both without effect in acute nephritis (Case I), so far as could be told by our observations. They were also without effect in Case IV in later observations, not shown in Text-fig. 2. We cannot at present make any general statements regarding the use of diuretics in nephritis, but the method offers opportunity for exact study of the action of various diuretic drugs when applied to diseased conditions.

#### *Relation of Chloride Excretion to Edema.*

We are not yet prepared to take up in detail the subject of the relation of chloride excretion to edema, but certain observations here presented refer also to this subject. Cases I, IV, and XI with nephritis; V, VI, and XII with heart failure; and XI with diabetes all showed edema, and all showed a relatively increased concentration of chlorides in the plasma. Case XIII (Text-fig. 6) had a pronounced generalized edema, occurring during serum sickness after pneumonia, after treatment with serum. Though his plasma chlorides had been low during pneumonia, they rose to a high point during the edema, and returned to the theoretical concentration as the edema disappeared. This case is of interest in that it illustrates two types of salt retention occurring in the same patient within a few days. During pneumonia, excretion was low, with a low concentration in the plasma. During serum sickness, excretion was again low, with a high concentration in the plasma. The normal period following the edema serves as a control for the other periods.



TEXT-FIG. 6. Case XIII. Lobar pneumonia; serum-treated. Crisis followed by serum sickness with generalized edema. Nov. 11. Urine, no albumin. Complete recovery occurred.

In Cases V, XII, IX, XI, and XIII disappearance of edema was accompanied by the return of the plasma chlorides to practically the theoretical figure. In Case I edema disappeared long before chloride excretion became normal, though the patient was constantly on a salt-poor diet. Case IV has never been free from edema and has always a relatively high concentration of chlorides in the plasma. Cases with persistent edema and low plasma chlorides have not been seen. Whether the relationship is one of cause and effect, or merely one in which the change in chloride excretion is due to the same cause as the edema, we cannot at present state.

## SUMMARY.

A method of measuring the rate of excretion of urea, in terms of the normal, as presented in a preceding paper, has been applied to the study of diseased conditions and of the action of drugs. Simultaneous observations on chloride excretion have been made by comparing the concentration of chlorides actually found in the plasma with the theoretical amount, calculated from the rate of excretion. Observations made in this way have been found applicable to the study of various diseased conditions.

## CONCLUSIONS.

1. The rate of excretion of urea in diseased conditions may be measured in terms of the normal by the index of urea excretion, and direct evidence as to the state of one of the more important excretory functions may be thus obtained.

2. The rate of excretion of chlorides gives a basis for calculating the theoretical concentration of chlorides in the plasma. By comparing the concentration actually found with the theoretical concentration, changes in the function of chloride excretion may be studied.

3. Increased concentration of urea in the blood is, as a rule, a compensatory phenomenon, in order to provide sufficient pressure to cause its excretion through a damaged outlet. Under certain conditions actual accumulation occurs.

4. Relatively increased concentration of chlorides in the plasma occurs in certain conditions, especially in certain forms of cardiac and renal disease.

5. Under certain conditions, notably in fevers or in diabetes, or as the action of diuretics (*digitalis*), the chloride threshold may be temporarily or permanently lowered. This may result in a decrease in the concentration of chlorides in the plasma to a point lower than the lowest point which is seen in normals.

6. Failure to excrete chlorides in pneumonia is associated with a lowered concentration of chlorides in the plasma. Excretion begins at the time this concentration increases.

7. Edema is usually accompanied by a relatively increased con-

centration of chlorides in the plasma. The relations ordinarily return to the normal state when edema disappears.

8. Chloride and urea functions may be quite independent of one another.

#### BIBLIOGRAPHY.

1. McLean, F. C., *Jour. Exper. Med.*, 1915, xxii, 212.
2. For a review of French work, see Ambard, L., *Physiologie normale et pathologique des reins*, Paris, 1914.
3. Widal, F., Ambard, L., and Weill, A., *Semaine méd.*, 1912, xxxii, 361.
4. Bauer, R., and Habetin, P., *Ztschr. f. Urol.*, 1914, viii, 353.
5. Bauer, R., and von Nyiri, W., *Ztschr. f. Urol.*, 1915, ix, 81.
6. Volhard, F., and Fahr, T., *Die Brightsche Nierenkrankheit*, Berlin, 1914, 168.
7. Schlayer, *Med. Klin.*, 1912, viii, Supplement, 211.
8. Widal, F., and Javal, A., *Compt. rend. Soc. de biol.*, 1904, lvi, 301.
9. Folin, O., Denis, W., and Seymour, M., *Arch. Int. Med.*, 1914, xiii, 224.
10. Frothingham, C., Jr., and Smillie, W. G., *Arch. Int. Med.*, 1915, xv, 204.
11. Peabody, F. W., *Jour. Exper. Med.*, 1913, xvii, 71.
12. von Fürth, O., *Probleme der physiologischen und pathologischen Chemie*, Leipzig, 1913, ii, 609.
13. A preliminary report of this portion of the work was published in the *Am. Jour. Physiol.*, 1915, xxxvi, 357.

## STUDIES IN INFANT METABOLISM AND NUTRITION, V.

UNDER THE DIRECTION OF L. EMMETT HOLT, M.D., AND D. D. VAN SLYKE, PH.D.

### THE COMPOSITION AND PREPARATION OF PROTEIN MILK (EIWEISSMILCH).

BY ANGELIA M. COURTNEY AND HELEN L. FALES.

*(From the Laboratories of The Rockefeller Institute for Medical Research and the Babies' Hospital.)*

The value of protein milk in disturbances of infantile digestion is appreciated in this country only by the comparatively small number who have used it extensively. It has not yet found its way into general practice and it is in general use in only a few of our hospitals. Many have been deterred from prescribing it owing to the difficulties in preparation as originally published. Much regarding the use of protein milk has been brought out since Finkelstein's original publication in 1910. A simplification and standardization of the method of preparation seems therefore desirable.

Not only a variation in the ingredients used but also the methods employed make noteworthy differences in the composition of the product. To these variations some of the differences in the results are no doubt due. Examination showed that samples of protein milk made in the same diet kitchen by the same nurse and with the same materials differed so widely, especially with respect to the fat content, that a careful study was undertaken to ascertain the effect of the different steps of preparation on the final product and to discover the explanation of these variations.

Finkelstein's<sup>1</sup> original method of preparation as described by him in his early publication is briefly as follows:

1. Finkelstein and Meyer: Jahrb. f. Kinderh., 1910, lxxi, 525.

To one liter of milk, one teaspoonful of rennet is added. This is then allowed to stand in a water bath at 42 C. for one-half hour. It is then placed in a linen bag and the whey strained off by suspending this for one hour. The curd is rubbed once or twice through a fine sieve with the addition of one-half liter of water, and one-half liter of buttermilk is then added.

The original milk used contained: fat, 3.5; sugar, 4.5; protein, 3.0; ash, 0.70. The protein milk contained: fat, 2.5; sugar, 1.5; protein, 3.0; ash, 0.50.

According to Finkelstein, the beneficial effects of protein milk were due to (1) its low sugar; (2) the dilution of the whey; (3) the high casein; (4) a combination of relatively high fat and high calcium, this favoring the production of formed stools.

Protein milk has been frequently described as a food low in salts; in fact, many writers have laid stress on the great reduction of the salts as one of the advantages of protein milk.<sup>2</sup> Rollet<sup>3</sup> proceeds on the assumption that it contains only the salts of the buttermilk which, according to Finkelstein's method of preparation, constitute one-half the fluid volume. Such, however, is not the case. In Finkelstein's and Meyer's own analyses the total salt content is five-sevenths of that of the original milk. But the proportion of the different salts is not the same as in whole milk; while the proportion of the soluble salts is reduced, that of the insoluble salts is increased.

Our analyses, which were first made in connection with metabolism studies, have always shown considerably higher values than those of Finkelstein and Meyer, not only for the total ash but for most of the ash constituents. This may have been in part due to the method of preparation which differed slightly from that of Finkelstein.

In the first place, in order to reduce the sugar content as much as possible, after the whey has been strained off we have washed the curd in sterile water. This was believed not only to reduce the sugar but also the salt content. The extent to which this is accomplished is shown in the analyses given in the subsequent tables.

Again, instead of adding one-half liter of water and one-half liter of buttermilk to the washed curd, only enough water was added to bring the final volume up to one liter, the difference being about 100 to 120 c.c. of water which is displaced by the curd.

2. Leopold: *Arch. Pediat.*, 1910, p. 602.

3. Rollet: *Berlin klin. Wchnschr.*, 1911, p. 835.

Finally, instead of the usual commercial buttermilk which contained about 4 per cent. of sugar, we used a specially fermented milk with but 3.5 per cent. sugar. This, moreover, was never fat-free but contained on the average 0.5 per cent. of fat; besides, there had been added a small amount of table salt. Our analyses showed, therefore, a higher NaCl content than that reported by Finkelstein.

*Variations in the Fat.*—Since samples of protein milk examined on three successive days gave the fat content as 2.2, 3.5 and 3.1 per cent., respectively, there were evidently conditions in the method of preparation which influenced the final product in an important way, and it was the purpose of our investigation to find out what these were, and to devise a method which reduced these variations to the minimum.

The causes of variation were apparently dependent on at least three different factors, any one of which might influence the percentage composition of the product, viz., (1) The percentage composition of the ingredients used. Although purchased from a large dealer the variation in the fat percentage in the whole milk from day to day was found to be over 0.5 per cent. (2) The conditions under which the curd was formed seemed important—the temperature, the time of standing, the amount of rennet added, the amount of the protein milk made at one time, etc. (3) The manner of manipulation in the straining, the washing of the curd and in the rubbing through the sieve, all had an effect on the composition of the product.

In order to decide these questions protein milk was made under varying conditions. It was prepared in different quantities varying from 1 to 10 liters at a time; the amounts of the elements used were accurately measured as well as the quantity of the final product. Complete analyses were made of the ingredients used—the milk, the buttermilk and the junket tablets; of the waste products—whey and washings; and of the final product, the protein milk. Analyses of the fat were made many times, this being the constituent which varied most. A smaller number were made of the protein, sugar and salts. It soon became evident that the chief causes of variation were to be found in conditions which affected the firmness of the curd. A firm curd held the fat more closely bound, and neither the fat nor the casein passed through the gauze in any appreciable amount;



while if the curd was soft, a considerable amount of both fat and casein were lost in the whey.

The routine procedure followed in the hospital diet kitchen was found to be as follows:

A quantity of whole milk, usually eight or ten liters, was heated to about 100 F. and into it was gently stirred for a moment one junket tablet, dissolved in a small amount of water, for each liter of milk. The milk was left standing, covered, at the temperature of the room, usually about 72 F., for thirty or forty minutes. It was then poured on two thicknesses of cheese-cloth, by tilting which the curd was gently rolled from side to side and the whey drained away in the course of eight or ten minutes. Water was then poured on the curd and the manipulation repeated. This washing was done twice. The curd was then placed on a fine wire sieve, fifty meshes to the inch, and rubbed through it with a metal vegetable masher, with the gradual addition of one-half liter of butter-milk to each liter of whole milk used for the curd. Enough water was then added to make the volume equal to that of the original milk.

It had long been a tradition of the diet kitchen that "good protein milk" could not be made from so small an amount as one or two liters of milk. Investigation confirmed this, for an analysis of the protein milk, made from one or two liters was far below the average in fat content, as can be seen from the following experiments.

From one lot of whole milk containing 3.3 per cent. fat, one-liter and four-liter samples were taken; from another lot containing 3.5 per cent. fat, one-liter, two-liter and four-liter samples were taken. Protein milk was made from each of these samples, the condition being kept as nearly as possible the same for all. The results are given in Table 1.

The figures showed extraordinary variation in the fat. That made from the first lot showed in the one-liter sample 2.15 per cent. fat; in the four-liter sample, 2.70 per cent. fat. In the second lot, the one-liter sample gave 1 per cent. fat; the two-liter, 1.6 per cent.; the four-liter sample, 2.37 per cent. (Vide analyses 1 to 5, Table 1.) In two ten-liter samples (Nos. 6 and 7) made from milk containing 3.6 per cent. fat the fat in the product was respectively 2.34 and 2.90 per cent. It would appear from this that the percentage of fat in the product is distinctly lower if only one or two liters, instead of a larger amount of milk, are used; but that above this quantity the exact amount used is not important.

TABLE I.  
*Variation in Fat of Protein Milk Due to Differences in Quantity Prepared and to Different Methods of Handling.*

No.	Quantity of Milk Used for Curd, c.c.	Method of Draining Whey from Curd	Grams in Milk Per Liter	Grams in Whey Per Liter	Grams in Wash Per Liter	Grams in Curd (By Subtraction)	Per Cent. of Amount in Original Milk in Curd	Grams in Buttermilk Per Half-Liter	Grams in Protein* Milk Per Liter (By Estimation)	Grams in Protein* Milk Per Liter (By Determination)
1	1,000	Entirely drained by handling in cheese-cloth.....	33	8.36	6.50	18.14	55.0	4.0	22.14	21.50†
2	4,000	Same.....	33	4.61	4.74	23.65	71.7	4.0	27.05	27.00
3	1,000	Same.....	35	18.35	7.00	9.75	27.9	5.5	15.25	10.00
4	2,000	Same.....	35	16.17	2.60	16.23	46.4	5.5	21.73	16.00
5	4,000	Same.....	35	12.28	1.73	20.99	60.0	5.5	26.49	23.70
6	10,000	Same.....	36	11.57	2.31	22.12	61.4	3.5	25.62	23.40
7	10,000	Same.....	36	9.00	.99	26.01	72.2	3.5	29.51	29.00
8	4,000	Same.....	35	19.26	2.40	13.34	38.1	4.0	17.34	11.00
9	4,000	Drained by suspending 2 hours.....	35	3.20	‡	31.80	90.9	4.0	35.8	35.00
10	3,000	Drained entirely by handling.....	35	12.91	1.26	20.83	59.5	4.5	25.33	24.00
11	3,000	Drained 10 minutes by suspension; rest by handling.....	35	6.35	1.73	26.92	76.9	4.5	31.42	31.00
12	3,000	Drained by suspending 2 hours.....	35	2.96	2.27	29.77	85.0	4.5	34.27	34.00
13	3,000	Drained entirely by handling.....	31	8.23	1.20	21.57	69.6	3.5	25.07	24.50
14	3,000	Drained 10 minutes by suspension; rest by handling.....	31	5.31	.99	24.70	79.7	3.5	28.20	28.00
15	3,000	Drained by suspending 2 hours.....	31	2.99	2.86	25.15	81.1	3.5	28.65	25.50
16	2,000	Drained by resting on sieve 10 minutes, then by handling.....	30	6.36	1.68	21.96	73.2	3.5	25.46	25.00

\* The amount of fat by estimation is necessarily higher than that obtained by determination because of the amount lost on utensils.

† The figures given in this column show the grams per liter; the per cent. is obviously one-tenth of this.

‡ By mistake this curd was not washed.

The reasons for the difference between large and small quantities seemed to be two: A certain amount of the fat always adhered to the cheese-cloth. In the one-liter samples the proportion of this to the whole amount of fat was considerable, while in the large samples the proportion to the whole was small. Again, in the manipulation of the curd used to remove the whey, the small curd broke apart more readily than the large curd, and consequently a larger proportion of the fat and casein were lost in the whey and in the washing. This made it clear that a method of removing the whey which involved less manipulation of the curd would insure a more uniform product and higher percentage of fat and of protein. This was demonstrated to be the case by the following experiments:

Two four-liter samples were measured from the same milk. The curd from one was handled in the usual way. That from the other was drained by suspension for two hours, the procedure in other respects being the same in both cases. From each of two other lots of milk three three-liter samples were taken. The curd in one sample in each group was drained by manipulating the gauze in the usual manner; that of another was suspended ten minutes and then manipulated till dry; that of the third was suspended for two hours without any manipulation. (Vide analyses 8 to 15, Table 1.)

In general these observations showed that the less the curd is manipulated in removing the whey, the higher the percentage of fat in the protein milk.<sup>4</sup> Of the four-liter samples the one in which the whey was removed by suspension showed 3.5 per cent., while the one manipulated showed but 1.1 per cent. One of the three-liter series gave the following values: Fat in sample suspended two hours, 3.4 per cent.; fat in sample suspended ten minutes and then manipulated, 3.1 per cent.; fat in sample manipulated without suspension, 2.4 per cent. The other series gave similar results. The apparent exception will be referred to later.

As a result of these observations the following procedure was adopted:

4. These experiments have proceeded on the assumption that a low fat was not generally desired in protein milk. The hospital experience has been that one of its chief advantages is its rather high fat content. However, should one wish to secure a low fat with high casein, skimmed milk, instead of whole milk, should be used in its preparation.

The whole milk is coagulated as above described. After standing for thirty minutes the coagulum is poured on a doubled piece of ordinary cheese-cloth and allowed to remain for fifteen minutes, the cloth resting on the sieve through which the curd is later to be pressed. The cheese-cloth is then gently manipulated for a few minutes to complete the removal of the whey. The curd is now washed twice, using each time about one-fifth as much water as the original amount of milk taken. The water is poured on the curd, which is then gently manipulated for two or three minutes. The curd is then transferred to the sieve and pressed through it with the gradual addition of half as much buttermilk as the volume of whole milk taken. Boiled water is then added to make the volume equal to that of the original milk used. Great care must be taken in pressing the curd through the sieve lest the fat be transformed into butter, which often adheres to the sides of the utensils or floats on the surface of the milk. This is avoided by rubbing gently in one direction, the rotary motion being almost certain to form butter. A fat determination in such a sample is manifestly unreliable. (Vide analysis 15, Table 1.)<sup>5</sup>

A series of over thirty observations (Table 2) was carried out to determine to what extent uniformity of fat content was secured with the method of handling the curd just described. On successive days samples of the original milk, the buttermilk and the protein milk were analyzed for fat. In protein milk made from the same milk supply by different persons there was a great variation in the amount of fat retained; it ranged between 52 and 86 per cent. of the fat in the original milk. (Vide analyses 1 and 2, Table 2.) When made by a single experienced person, not only was the fat in the protein milk more nearly uniform but the actual loss of fat was much less. In one instance, the range of fat retained was from 86 to 97 per cent. of the fat in the milk; in another, 76 to 79 per cent.; in a third, 93 to 97 per cent. (Vide analyses 3 to 5, Table 2.)

From these figures the proportion of the fat of the whole milk retained in the curd and consequently in the protein milk is easily estimated. With proper handling fully four-fifths of the fat of the whole milk used is retained in the protein milk. But the total fat of the protein milk contains also a small amount of fat in the buttermilk. If commercial buttermilk is used, however, this is a negligible quantity. Consideration of the figures given in the foregoing table forces one to the conclusion that a very important factor in insuring a uniform fat in protein milk is the care used in the various steps of the operation.

5. The loss of fat in whey and washings indicates that the fat value of the protein milk is too low.

TABLE 2.  
*Variations in the Fat of Protein Milk When Prepared According to a Proper Method, by Different Persons.*

No.	No. of Determinations Considered	By Whom Prepared	Range of Fat Per-centage in Whole Milk Used	Average Fat Per-centage of Fat of Whole Milk	Range of Fat Per-centage of Protein Milk	Average Per Cent. of Fat of Protein Milk	Range of Per Cent. of Whole Milk Fat Held in Curd	Average Per Cent. of Whole Milk Fat Held
1	10	Prepared by various persons.....	3.00-3.65	3.37	0.35-0.75	0.55	61-86	74.2
2	10	Prepared by various persons.....	3.15-3.40	3.29	0.40-1.70	0.68	52-83	73.6
3	6	Prepared by Nurse A.....	3.00-3.30	3.10	0.5-0.6	0.57	86-97	90.6
4	4	Prepared by Nurse B.....	3.00-3.90	3.44	0.65-0.90	0.79	76-79	78.0
5	2	Prepared by Nurse C.....	3.00-3.00	3.0	0.8-0.8	0.80	93-97	95.0

TABLE 3.

*Average Composition (Fat, Sugar, Protein, Total Ash) of Protein Milk, of the Ingredients Used, and of the Whey and the Wash (From Five Analyses).*

	Grams in Milk Per Liter	Grams in Whey Per Liter	Grams in Wash Per Liter	Grams in Curd (By Subtraction)	Per Cent. of Amount in Original Milk in Curd	Grams in Buttermilk Per Half-Liter	Grams in Protein * Milk Per Liter (By Estimation)	Grams in Protein * Milk Per Liter (By Determination)
Fat.....	34.0	5.65	2.14	26.21	77.1	4.0	30.21	29.8
Sugar.....	46.7	41.53	4.89	0.28	0.6	18.06	18.34	22.0
Protein.....	31.9	7.71	1.62	22.57	70.8	15.36	37.93	37.5
Total Ash.....	7.934†	4.751	0.597	2.586	32.6	4.034	6.620	6.528

\* The values for the ingredients of protein milk obtained by estimation are necessarily greater than those obtained by determination because of the amount lost on utensils.

† The salt of the junket tablets used in coagulating the milk is included in the total ash of the milk.

The method of preparation which has been described above is the one to be recommended where considerable quantities of protein milk are to be made at one time, as for hospital use. If only one or two liters are desired, or it is to be made by an inexperienced person, the chances of error are much less if all the whey is drained away by suspension of the curd.

The other constituents—sugar, protein and salts—as has been already suggested, vary much less than does the fat.

*The Sugar.*—The sugar content of the original milk used ranged from 4.5 to 4.8, the average being 4.7 per cent. When the protein milk is properly made there is removed in the whey and in the washing nearly all the sugar of the original milk. On the average, about nine-tenths of what is removed comes out in the whey, and one-tenth, in the washing. The percentage of sugar in the protein milk should therefore be only about one-half that in the buttermilk. The figure for sugar in all our analyses was obtained by the reduction method. Owing to the difficulty of removing the protein from the protein milk before determining the sugar value, the figure for sugar is probably too high. Our average sugar figure in protein milk made under proper conditions is about 2 per cent. This figure is obtained from a larger number of determinations than are included in Table 3. The apparent error, assuming the curd to be nearly sugar-free, is therefore, 0.2 per cent. The actual sugar content of protein milk carefully prepared, as above described, must average about 2 per cent., using commercial buttermilk, or a little less than 1.8 per cent., using the specially fermented milk.

*The Protein.*—The percentage of protein in the protein milk is influenced by the same conditions as those which affect the fat, but to a less degree. The protein of the whole milk used averaged 3.25 per cent. When properly made there is lost in the whey 0.77 per cent.; in the washings, 0.16 per cent. Besides the lactalbumin, this must include some of the casein mechanically carried through. Some is also left on the utensils. The protein of the buttermilk used averaged 3.07 per cent. The total percentage of protein of the product would be half this (since the buttermilk represents one-half the volume) plus the curd protein which, after deducting the loss in the whey and the waste, leaves about 2.26 per cent. of the original milk.

By addition this would make the protein content of the protein milk 3.79 per cent.; by determination it was 3.75; the range being 3.60 to 4.00 per cent. Of this protein only about 0.25 is lactalbumin,

TABLE 4.

*Distribution of the Salts of the Ash of Protein Milk, of the Ingredients Used, and of the Whey and the Wash (From Five Analyses).*

	Grams in Milk Per Liter	Grams in Whey Per Liter	Grams in Wash Per Liter	Grams in Curd (By Sub- trac- tion)	Per Cent. of Amount in Original Milk in Curd	Grams in But- ter- milk Per Half- Liter	Grams in Pro- tein * Milk Per Liter (By Es- timation)	Grams in Pro- tein * Milk Per Liter (By De- termination)
CaO.....	1.757	0.486	0.108	1.163	66.2	0.833	1.996	1.930
MgO.....	0.199	0.101	0.015	0.083	41.7	0.104	0.187	0.180
P <sub>2</sub> O <sub>5</sub> .....	2.060	0.796	0.140	1.124	54.6	1.024	2.148	2.121
K <sub>2</sub> O.....	1.887	1.514	0.167	0.206	10.9	0.828	1.034	1.028
Na <sub>2</sub> O.....	0.770†	0.636	0.070	0.064	8.3	0.511	0.575	0.563
Cl.....	1.420†	1.148	0.120	0.152	10.7	0.764	0.916	0.868

\* The values for the ingredients of protein milk obtained by estimation are necessarily greater than those obtained by determination because of the amount lost on utensils.

† The NaCl of the junket tablets is included in that of the milk.

TABLE 5.

*Comparison of the Salts of Different Milks.*

	Total Ash, Per Cent.	CaO	MgO	P <sub>2</sub> O <sub>5</sub>	K <sub>2</sub> O	Na <sub>2</sub> O	Cl
Protein milk (12 analyses).....	0.648*	0.201	0.021	0.222	0.109	0.032*	0.061*
Cow's milk (5 analyses).....	0.743	0.176	0.020	0.206	0.189	0.050	0.111
Woman's milk (16 analyses of normal mature milk).....	0.206	0.047	0.008	0.034	0.057	0.014	0.035

\* This figure is obtained after subtracting for the NaCl added to buttermilk used in our experiments.

about 3.50 being casein. By the method of preparation—precipitation and mechanical subdivision—this large amount of casein is readily held in suspension and passes through the ordinary rubber nipple. If carefully warmed for feeding this suspension is not interfered with. But owing to the acidity of the milk any considerable degree of heat causes a separation and precipitation of the casein in large masses.

*The Salts.*—So much has been said of the salts in the literature of protein milk that the total salts and the distribution of the salts were made a subject of special study. In general it was found, as was anticipated, that the soluble salts of the original milk were largely removed in the whey and in the washings, while a large part of the insoluble salts was retained in the curd.

There is retained in the curd 62.5 per cent. of the CaO of the whole milk

"	"	"	"	39.9	"	"	"	MgO	"	"	"
"	"	"	"	53.5	"	"	"	P <sub>2</sub> O <sub>5</sub>	"	"	"
"	"	"	"	10.7	"	"	"	K <sub>2</sub> O	"	"	"
"	"	"	"	6.4	"	"	"	Na <sub>2</sub> O	"	"	"
"	"	"	"	7.2	"	"	"	Cl	"	"	"

The salts of the final product will therefore be the salts of the buttermilk plus those retained in the curd. In Table '4 is shown the quantity of each of the salts in the milk and buttermilk used, also the amount removed in the whey and the washings and the amount present in the protein milk.

In Table 5 are shown the salts of protein milk compared with those of cow's milk and woman's milk.

It will be noted that the proportion of CaO and P<sub>2</sub>O<sub>5</sub> in protein milk is slightly higher than in cow's milk while that of MgO is practically the same in both. A reduction is seen only in K<sub>2</sub>O, Na<sub>2</sub>O and Cl, the amount of which is but little over half as great as in whole milk.

As compared with woman's milk, not only are the total salts of the ash in great excess, but the amount of calcium is nearly five times, and the phosphorus, nearly seven times as great. The soluble salts also are nearly twice as abundant in protein milk.

To the large excess of insoluble salts, especially calcium, is no doubt to be ascribed the value of protein milk in producing formed stools.

From these observations there is found no basis for the statement that protein milk is low in salts, since it contains not only a higher total ash, but higher amount of all the different salts than are ordinarily given to infants artificially fed. Even when protein milk is diluted, the amounts are much greater than nursing infants receive. The advantages of its use cannot therefore be attributed to the



amount of salts removed, nor can the failure to gain weight be ascribed to the absence of the usual salts of the whey. The lowering of the  $\text{Na}_2\text{O}$  and  $\text{Cl}$  in the preparation of the protein milk seems to be of no special advantage. As used in the Babies' Hospital extensively for three years with most satisfactory results, it has contained, owing to the addition of  $\text{NaCl}$  to the buttermilk used, an amount of these elements nearly as great as in cow's milk and much greater than that in woman's milk.

#### CONCLUSIONS.

1. The chief variation in the composition of protein milk is in the fat. Uniformity is secured not only by the use of ingredients of uniform composition, but, what is much more important, by the exercise of great care in the handling of the curd. It should be suspended for a short time or allowed to rest for fifteen minutes on the sieve before manipulation to drain off the whey; care in pressing the curd through the sieve is also essential.

2. Since the value of protein milk is in large measure due to its low sugar content, the washing of the curd with water is a useful means of removing an additional amount of sugar.

3. When properly prepared the amount of protein in protein milk is quite constant and is usually somewhat greater than that of the original milk; it is nearly all casein.

4. If the buttermilk is added while the curd is being rubbed through the sieve, it is unnecessary to repeat this part of the process.

5. In the ash of protein milk the amount of calcium and phosphorus is slightly greater, that of sodium, potassium and chlorine is less than in whole milk, being reduced to a little more than one-half the proportion present.

6. Certain things are to be avoided in preparing protein milk: (1) stirring too much or too long while adding the rennin; (2) leaving the milk during curd formation in a cold place; (3) any unnecessary handling of the curd in straining off the whey or in washing or in pressing through the sieve; (4) subsequent heating beyond that required in feeding.

7. The composition of protein milk obtained from all our analyses

when made as above described is: Fat, 3.0 to 3.50 per cent.; sugar, 1.8 to 2.0; protein, 3.60 to 4.00; ash, 0.65.

#### REFERENCES.

In addition to the authors referred to in the text, the following may be consulted:

Pelka: *Ztschr. f. Kinderh.*, ii, 442.

Various modifications of protein milk:

Stoelzner: *München. med. Wchnschr.*, 1913, vi, 291.

Heims: *Monatschr. f. Kinderh.*, 1913, xi and xii, 621.

Bertlich: *Ztschr. f. Kinderh.*, 1913, Nos. 3, 5, 9.

Engel: *Deutsch. med. Wchnschr.*, 1913, p. 26.

Kern and Müller: *Berlin klin. Wchnschr.*, 1913, p. 2237.

Hoobler: *Arch. Pediat.*, 1914, p. 171.

## RESULTS OF APPLYING A QUANTITATIVE METHOD TO THE ABDERHALDEN SERUM TEST FOR CANCER.

BY ISAAC LEVIN, M.D., AND DONALD D. VAN SLYKE, PH.D.

*(From the Department of Cancer Research of the Montefiore Home and the Hospital of The Rockefeller Institute for Medical Research.)*

In a previous publication, one of the writers<sup>1</sup> reported a study of the value of the Abderhalden test for cancer, a comparative investigation with the dialysis method in cases of carcinoma, sarcoma and tuberculosis. As a substrate, carcinoma tissue was used for all the cases. Of thirteen carcinoma cases, twelve were positive and one negative; the sarcoma cases were all positive. Of twenty tuberculosis cases, nine were positive and eleven negative. The analysis of the results showed that the Abderhalden dialysis method cannot be considered as yet of real diagnostic value, since it is positive in nearly 50 per cent. of non-cancerous patients.

Before further investigations could be attempted in regard to the question of the specificity of the reaction and the correctness of Abderhalden's theoretical conceptions, it seemed imperative to find a more accurate test for the detection of the ferment actions of the blood serum. Van Slyke and Vinograd<sup>2</sup> reported a year ago on a quantitative application of the Abderhalden serum test.

Their technic was the following: Two c.c. of serum are digested with 0.1 gm. of dried substrate (tissue prepared according to Abderhalden's directions and dried at 0.5 mm.). After the digestion is complete, 3 c.c. of water are added. The solution is then centrifuged, and 2 c.c. used for aminonitrogen determination in the apparatus of Van Slyke. Control analyses are run under the same

1. Levin, Isaac: Proc. New York Path. Soc., 1914, xiv, 115.

2. Van Slyke, Donald D., and Vinograd, Mariam: Proc. Soc. Exper. Biol. and Med., 1914, xi, 154.

conditions with serum that has been digested with normal tissue and without any tissue. The occurrence of proteolysis is shown and its extent measured by the increase in aminonitrogen. As the amino-nitrogen is transformed into elementary nitrogen gas, which is measured in a buret, the results are both quantitative and free from subjective influence.

TABLE I.—*First Series.*

No. of Case	Condition	Increase in Nitrogen Gas from 1 c.c. Serum c.c.	Dialysis Test. Ninhydrin Reaction
1	Normal	.07	.....
2	Normal	.11	.....
3	Pregnant	.24	.....
4	Pregnant	.16	.....
5	Normal	.10	.....
6	Normal	.00	.....
7	Normal	.00	.....
8	Normal	.18	.....
9	Carcinoma	.22	+
10	Carcinoma	.20	+
11	Carcinoma	.28	+
12	Carcinoma	.00	+ +
13	Carcinoma	.29	+ + +
14	Carcinoma	.22	+ + +
15	Carcinoma	.29	+ + +
16	Carcinoma	.06	.....
17	Carcinoma	.08	+ + +
18	Carcinoma	.04	+
19	Carcinoma	.01	+ + +
20	Carcinoma	.25	+ + +
21	Carcinoma	.44	+ + +
22	Carcinoma	.24	+ +
23	Carcinoma	.14	+ + +

This method undoubtedly obviates a great many of the possible errors inherent in the dialysis method, and it was therefore decided to employ it for the purpose of further investigation of the value of the Abderhalden test for the diagnosis of cancer.

In the first series (Table I), the test was used in eight normal cases and on fifteen carcinoma patients. In all twenty-three cases the same carcinoma substrate was used. The tissue was taken from a patient with adenocarcinoma of the breast. It was prepared in accordance with the rules laid down by Abderhalden and then dried. Table I shows the increase in nitrogen gas from 1 c.c. of blood serum. An analysis of the table shows that, with the exception of

Case 21, the increase varies over nearly the same range in the normal as in the carcinoma cases, though the average increase is somewhat greater in the carcinoma cases. In every carcinoma case a parallel test was also made by the aid of the dialysis method. The same table shows the result. The sign + means a faint color reaction, ++ a stronger and +++ a very strong reaction; 0 means a negative reaction. The dialysis results do not seem to coincide with the amount of increase in the aminonitrogen. Case 12, for example, showed no increase in the aminonitrogen, but was positive by the dialysis method.

TABLE 2.—*Second Series.*

No. of Case	Condition	Increase in Nitrogen Gas from 2 c.c. Serum c.c.	Dialysis Test, Ninhydrin Reaction
1	Normal	.10	.....
2	Normal	.21	.....
3	Normal	.06	.....
4	Normal	.16	.....
5	Normal	.11	.....
6	Normal	.10	.....
7	Normal	.19	.....
8	Normal	.18	.....
9	Carcinoma	.10	+++
10	Carcinoma	.14	.....
11	Carcinoma	.05	.....
12	Carcinoma	.07	.....
13	Carcinoma	.08	+
14	Carcinoma	.00	0
15	Carcinoma	.08	+
16	Carcinoma	.13	+
17	Carcinoma	.28	++

After these results had been obtained, it was found that the accuracy of the aminonitrogen determination could be increased by removing the proteins of the serum with colloidal iron before the aminonitrogen determination was performed.

The method thus improved was employed in another series of cases. The blood serum of eight normal individuals and nine carcinoma cases was tested, using for substrate the same carcinoma tissue which was employed in the first series. Table 2 shows the result of this series. Again the increase in the nitrogen gas formation varies within the same limits in the normal as in the carcinoma-

atous serum. In this series the average increase of nitrogen gas seems to be greater in the normal cases than in the carcinomatous. The blood serums of six carcinoma cases were also tested by the dialysis method, while in the other three cases a sufficient amount of serum could not be obtained for the double test. Here again the strength of the color reaction does not coincide with the lesser or greater quantity of nitrogen gas. Case 14 is interesting inasmuch as it was negative with both tests.

The analysis of the results of the present investigation seems to indicate that the more accurate and objective the test employed for the detection of the specific ferment reactions in the blood serum, the less difference can be detected between the reactions obtainable with normal and supposedly specific serums. The diagnostic value of the Abderhalden reaction in cancer is, therefore, to say the least, doubtful, and it must be stated very emphatically that for the present the method belongs to the research laboratory and not to the clinic.

## METABOLIC STUDIES IN DIABETES.\*

By FREDERICK M. ALLEN, M.D.

*(From the Hospital of The Rockefeller Institute for Medical Research.)*

In the work with diabetes at the Rockefeller Hospital, the principal problem has been one of treatment, rather than the study of metabolism as such. But the treatment has been based upon the conception of diabetes as the weakness of a metabolic function, and opportunity for some new observations concerning diabetic metabolism has been afforded by the method which clears up the symptoms of severe cases more quickly and radically than has been the practice heretofore.

In animals, total removal of the pancreas gives rise to an acutely fatal form of diabetes which differs in some respects from the form seen in human patients. But partial pancreatectomy produces a closer imitation of the clinical conditions.<sup>1</sup> The effect varies according to the amount of pancreatic tissue removed. There may be a simple lowering of the sugar tolerance without diabetes. Or, diabetes may be produced by sugar and prevented by omitting sugar from the diet. Again, diabetes may be produced by starch, and be prevented by partial or complete withdrawal of carbohydrate from the diet. Again, diabetes may be present on unlimited protein diet, and be checked by a few fast-days and restriction of the quantity of protein. In a still more severe form, the diabetes is not thus controlled, and the animal passes into a fatal condition if these half-way measures are persisted in. Such an animal can be made sugar-free by a fast sometimes lasting weeks rather than days. It can then be kept thin and free from glycosuria on a very low diet. Increase of diet or body weight tends to bring back the glycosuria. The addition of fat to the diet can be shown to have this effect in suitable

\* Read at the Annual Meeting of the Medical Society of the State of New York, at Buffalo, April 28, 1915.

animals. Any influence which causes glycosuria, in any of the above types of animals, gives rise to a sequence of lowered tolerance, loss of weight, weakness, cachexia and death. Thus the course resembles that of a progressive fatal disease. But what is actually present is known to be the lowered functional power of a certain organ, produced by surgical removal of the greater portion of that organ. When this weakened function is not overtaxed, any of the above types of animals can be kept indefinitely free from downward progress, and they generally tend rather to improve slightly with time.

The existence of a large class of patients, whose diabetes is so severe that the glycosuria and acidosis are not abolished under any known mode of treatment, is familiar to all physicians, and is recognized by all authorities on this subject and not disputed by any writer so far as I have ever seen. Naunyn, who was the great pioneer in the use of occasional single or repeated fast-days and slight temporary undernutrition, recognized that stopping the glycosuria was the indispensable condition of checking the downward progress, but also admitted that in many cases this was impossible. Von Noorden and other writers are perfectly clear in similar statements. Magnus-Levy<sup>2</sup> forbids fast-days oftener than once in eight or ten days, and in very emaciated patients he opposes fasting altogether. Foster<sup>3</sup> describes the benefits of rigidly restricted diet and occasional fast-days singly or in series, the necessity of months of such treatment to rid the urine of sugar in advanced cases, and the general unfavorable prognosis for severe diabetes and the inevitable death in coma at last.

Concerning the application of the results obtained with animals to the radical treatment of human diabetes, the following points may be mentioned.

First, it seems possible, broadly speaking, to stop glycosuria promptly in any case of human diabetes. As much as eight or ten days of continuous fasting may be necessary for this result. Even very weak and emaciated diabetics have endured this program with apparent benefit. Infections and some other complications seem to delay the clearing up of the glycosuria. Contraindications have not been met, unless rarely if the fasting patient shows nausea and



vomiting. It may then be best to terminate the fast and give restricted diet for a week or two, after which another fast easily abolishes the glycosuria. In one very severe case in a boy of seventeen, studied by Dr. Eugene DuBois in the respiration calorimeter, he found that the total metabolism was about eight per cent below normal, and the patient was excreting all or practically all of the sugar formed from protein, and burning practically no sugar at all. In a nine days' fast, the glycosuria ceased, the total metabolism fell to about thirty per cent below normal, and the respiratory quotient showed that the sugar formed from protein was being burned.

Also, acidosis can apparently be controlled in even the severest cases. Fasting produces a slight excretion of acetone bodies in normal human beings, and it may seem a metabolic curiosity that fasting, without carbohydrate, should so markedly diminish diabetic acidosis. There is reason for believing that normal persons vary considerably in the ketonuria produced by protein-fat metabolism on deprivation of carbohydrate. Diabetics show similar, possibly greater variations in this respect; and the intensity of the diabetes or of the acidosis is not a reliable index of the stubbornness of the ketonuria. Some patients with apparently fairly mild diabetes, whose acidosis was never alarming and who easily become and remain sugar-free, still keep up a persistent ketonuria with a marked ferric chloride reaction on fasting, and on a diet low in fat and protein and containing an appreciable quantity of carbohydrate. This condition may be observed especially in the more obese patients. On the other hand, some patients with the severest diabetes, with extreme emaciation and with coma threatened or actually present, may show a negative ferric chloride reaction by the end of the long initial fast or within a few days thereafter; and they may then go along excreting mere traces of acetone bodies on strictest carbohydrate-free diet. It is possible that such patients have a naturally high power of burning acetone bodies, or that they developed an unusual power of this sort during their period of intense acidosis. The ideal treatment is to abolish ketonuria as well as glycosuria. If some patients continue to show the same physiological trace of ketonuria that a normal person might show on similar restriction of carbohydrate, this cannot be changed until continued treatment has

raised the carbohydrate tolerance. But the procedure has been to keep on with fasting, with periods of very low diet, and with other periods of nothing to eat but green vegetables up to the limit of carbohydrate tolerance, as long as may be necessary to remove acidosis. It has been the rule not to increase the fat of the diet, or to begin building up the patient's weight and strength, until this control of the acidosis has been achieved. Under this program, the ferric chloride reaction has been made negative in every patient who has been under treatment for the necessary length of time.

A slight reduction of the patient's weight at the beginning of treatment in certain cases has been the practice of Naunyn and some later clinicians. But in general, these men as well as others have attempted to build up the weight and strength as much as possible, with the purpose of making the patient better able to withstand a wasting disease. It is unquestionable that loss of weight due to the diabetes itself, with continuous glycosuria and acidosis, is an unfavorable symptom, as has always been held. But the reduction of weight by fasting and low diet, so as to abolish glycosuria and acidosis, is an altogether different matter, and is an important initial step in the treatment, especially of severe cases. It is believed that this reduction of weight is in itself beneficial to the diabetic condition. It is not limited to the few pounds previously ventured, but is carried to any point that may be necessary to remove glycosuria and acidosis. In some instances a small reduction of weight suffices for even a severe case. On the other hand, one well-nourished patient, whose urine was easily freed from sugar, was reduced in weight to the extent of twenty kilograms, merely because of a slight stubborn ketonuria and a persistently high blood-sugar. Also, the reduction in weight is not temporary, as was the former practice. The patient is allowed to gain weight and strength, provided he can do so without glycosuria or acidosis. If he cannot gain on these terms, he is not allowed to gain at all. As a matter of fact, almost every patient does become able to gain to some extent. This power of recuperation varies widely in different patients, and the apparent severity of the case is no safe criterion as to how fast or how far the lost function may return. But no patient is allowed to return fully to his original normal weight. He is required, by means of

fasting and reduced diet, to keep himself below a certain number of pounds, which is prescribed on the basis of observations during his stay in the hospital. Anyone can easily convince himself that, in a severe diabetic who remains free from symptoms at a suitably low weight, both glycosuria and ketonuria can frequently be brought back by any attempt to raise the weight too high. Probably many failures in treating severe diabetes by the previous methods are due to this mistake; and the customary gloomy prognosis, and the belief that every severe diabetic relapses sooner or later, may perhaps be explained by therapeutic methods which have tried to maintain patients on carbohydrate-free diet at too high a level of weight.

The initial fast, to clear up glycosuria and diminish acidosis, generally presents less difficulty than the subsequent diet which must keep the urine normal and yet support life. Such a regimen has been sketched briefly in previous papers;<sup>4</sup> but the treatment should be carefully individualized to suit the needs of each patient, and details can be given only in a more complete publication to appear after the cases have been followed for a longer time. The difficulties of a severe case may tempt the physician to conclude that his patient cannot be kept sugar-free; that it is not possible on any diet. Certainly knowledge, care, and strict control are necessary in the management of any severe case, and undernutrition may be dangerous and harmful if wrongly employed. A skilled diet nurse is an important aid. The number of patients treated at the Rockefeller Hospital has been forty-two. Applications have constantly exceeded the accommodations, and choice has been made of the severest ones, with special preference for young, acute cases with coma threatened or present. When the glycosuria has been rapidly cleared up by the initial fast as described, it has subsequently been possible to keep the patient sugar-free in every case thus far. One feature of the diet in all severe cases is the restriction in fat and calories. Restriction of carbohydrate was the first dietetic treatment of diabetes. Restriction of protein has been practiced for years. But fat has ordinarily been forced upon the diabetic, and the best cook has been considered the one who could get most fat into the patient. Exceptions to the rule have been rare,—chiefly temporary limitations for patients threatened with coma. The prevalent doctrine has been

that the diabetic must receive the number of calories required by his metabolism, plus the number of calories lost as sugar and acetone bodies in the urine; and the best food for crowding in these calories has been supposed to be fat. There have been quacks who declared that as the diabetic loses sugar in the urine, this ought to be replaced by means of sugar in the diet. The accepted idea of replacing lost calories may be compared to this notion of replacing lost sugar. Both methods, in different ways, break down the weakened metabolic function and send the patient downhill. The opposite course is to clear up glycosuria and acidosis, and give the patient no more food than he can metabolize properly. The prevalent doctrine has been that fat feeding does not affect diabetic glycosuria, unless in a few "fat-sensitive" patients. The effect of fat is masked by the glycosuria and other symptoms present in the severest cases of diabetes under ordinary treatment. But when these symptoms have been cleared up, the influence of fat can be easily demonstrated. No clinical experiment is more simple and definite than to take a suitably severe diabetic who is symptom-free for days or weeks on a fixed diet, and to observe how the addition of certain quantities of butter or olive oil to this diet brings back the glycosuria, ketonuria and subjective symptoms immediately or within a brief time. The feeding of fat without other food does not cause glycosuria, and these experiments do not serve as evidence of the formation of sugar from fat. But they do show that forcing the body to dispose of fat beyond a certain quantity injures its power to dispose of carbohydrate or protein; and they thus perhaps indicate further causes of failure in the treatment of diabetes in the past.

Alcohol in the form of whiskey or brandy is generally given during fasting and often for a certain period thereafter, since in reasonable doses it does not cause glycosuria, and Neubauer<sup>5</sup> and Benedikt and Török<sup>6</sup> claimed that it diminishes acidosis. Its prolonged use is nearly always avoided.

Patients are kept comfortably warm at all times, and generally at rest in bed during the initial fast and sometimes during the period of minimum diet. Exercise without undue excitement is permitted in later periods. Its effect is still under investigation.

Since the new Benedict-Lewis method<sup>7</sup> affords a means for the

accurate determination of the blood-sugar in ten minutes, this method has been used for frequent analyses, which show that the blood-sugar in some of the most serious cases has been brought rather quickly to normal. In other cases still under treatment, the hyperglycemia is stubborn, and further observations may show whether it is feasible or essential to insist on a normal blood-sugar in every case.

Drs. Van Slyke, Cullen and Stillman<sup>8</sup> have investigated the relations between carbon dioxide tension in the alveolar air, carbon dioxide capacity of the blood, reserve alkalinity of the blood (measured by the hydrogen ion concentration after adding known quantities of acid), and clinical manifestations of acidosis. The parallelism between these various findings proves to be very close. Van Slyke's method for the carbon dioxide of the blood requires only ten minutes' time and rather simple laboratory equipment. The Fridericia<sup>9</sup> instrument for the alveolar air can be used at the bedside by any physician as easily as a blood-pressure apparatus; and it also seems to afford a reliable index of the acidosis. The blood and respiratory analyses have confirmed the clearing up of acidosis under treatment.

Finally, the clinical and subjective condition of the patients is a natural subject of inquiry. No patient has yet refused to take the treatment. Even very weak and emaciated patients have borne the treatment well. Of forty-two patients, seven are dead. In view of the character of the cases taken, this record is satisfactory, for it includes not only coma cases but also pulmonary, cardiac, and other conditions aside from the diabetes. Two patients were discharged for violations of discipline not pertaining to diet; one of these is included in the above list of deaths. The other patients are free from glycosuria, and are subjectively improved in greater or less degree. Those leaving the hospital are taught to test their urine with Benedict's solution<sup>10</sup> and to manage their diet and weight so as to keep sugar absent. Relapses thus far have not been numerous or serious, the longest period of observation being fourteen months. No complication has appeared in any patient under treatment. Existing conditions such as excessive hunger and thirst, pruritus, neuralgia, and functional eye troubles have disappeared. Carbuncle

and gangrene (one case each) have likewise cleared up promptly, and operative or other dangerous complications are considered an indication for the radical treatment. Albuminuria has cleared up in some cases; in other cases with possible organic kidney trouble it has not cleared up. No patients have needed alkali for more than a few days. Gastro-intestinal troubles are absent. The intense craving for carbohydrates on the part of diabetics has probably in the past been due to the over-heavy protein-fat diet, and this has given rise to the unjust opinion that diabetic patients cannot be trusted. At first there is the natural hardship of breaking up a person's established habits as respects both kind and quantity of food. But the patients have quickly become contented on their new diet and have followed it conscientiously because of the improvement which they feel. Even those whose weight has been markedly reduced feel improved strength and well-being. The degree and rapidity of improvement up to the present has varied widely in different cases. Exceptional patients have, from a condition of serious danger, come rapidly back to the feeling and appearance of perfect health, with merely the inconvenience of care in diet. Exceptional patients at the other extreme have shown little recuperative power. Some of these latter remain thin and weak because the diet must be kept low. Others of this class complain of weakness and other symptoms, though the diet is theoretically adequate and the nutrition apparently good. The chief benefit to these patients is that they now seem to be holding their own or slightly improving, whereas previously they were going downhill. The majority of the results fall between these two extremes. The majority of patients have been decidedly improved in strength and well-being, but have not been able to regain fully normal weight or strength. In some of them, the improvement seems to go on continuously. In others it is at a standstill, or is so slow that years will be necessary for appreciable gain. The encouraging feature is that the average tendency seems to be more upward than downward.

The principal theoretical question involved is whether diabetes is an inherently progressive disease, or whether it is the simple weakness of a metabolic function. If it is the former, the patients must go downhill ultimately, just as ordinarily predicted for severe dia-

betics, and the only benefit of treatment will be to prolong their lives and make them more comfortable. If it is the latter, downward progress may be indefinitely prevented by avoiding over-strain of the weak function, just as in animals. Previous experience in the treatment of diabetes does not decide this question. For one thing, the proposed new treatment differs from the old in being more radical and thorough, and the difference between the partial and the complete clearing up of a diabetes may be comparable to the difference between the partial and the complete extirpation of a carcinoma. For another thing, the proposed treatment directly reverses the previous practice in certain points; in particular, it contradicts the prevalent beliefs that the diabetic should be fed up to keep his weight high, and that calories lost in the urine should be replaced in the diet, and that unlimited fat feeding is beneficial and does not influence diabetic glycosuria. The severest cases may not be suitable for a decisive test. The function in some of these patients may be so weak that an over-strain is involved in keeping them alive at all, and thus they may finally progress downward. Time and further experience alone can decide. Meanwhile, the true opinion is not that of the pessimist who considers everything useless, nor that of the optimist who expects miracles, but it seems to lie somewhere between. Granted that diabetes is merely the weakness of a metabolic function and its most dangerous symptoms can be rapidly removed by relieving over-strain, yet the fact of this weak function must be faced. The patient has, so to speak, a defective boiler, and it seldom again becomes able to stand a full head of steam. The best prognosis in diabetes, and the greatest reduction of the mortality, will come when the average general practitioner takes the methods which have proved their value in severe diabetes, and applies them so as to control the cases that are yet early or mild. In such cases there is cause to hope that prompt thorough treatment will save the weakened function before it becomes too weak, and that the patient may then go along on moderately guarded diet and slightly reduced weight, with not only his life but also his usefulness preserved. The most advanced and distressing stages of a number of medical and surgical conditions, which were common enough in former times, are now rarely seen, because treatment is applied in

the earlier stages. The number of patients who are seen in the last, most desperate stage of diabetes is still very large, and the most hopeful prospect for therapy cannot lie in attempts to cure patients at this stage. It is to be found rather in a more general recognition of the duty of stopping glycosuria and the feasibility of doing so, in better instruction of patients, and in the use of thorough and efficient measures at the earliest possible stage of diabetes.

#### LITERATURE.

1. Allen, F. M.: *Glycosuria and Diabetes*, Harvard University Press, Cambridge, Mass., 1913.
2. Magnus-Levy, A.: *Spezielle Pathologie und Therapie innerer Krankheiten* (Kraus and Brugsch), 1913, p. 67.
3. Foster, N. B.: *Diabetes Mellitus*, Lippincott & Co., 1915, p. 215 ff.
4. Allen, F. M.: *Journal of the American Medical Association*, September 12, 1914, p. 939; *Boston Medical and Surgical Journal*, February 18, 1915.
5. Neubauer, O.: *Münch. Med. Wochenschrift*, 1906, 791-793.
6. Benedikt and Török: *Ztsch. für klin. Medizin*, 1906, LX, 329-348.
7. Lewis, R. C., and Benedict, S. R.: *Journal of Biological Chemistry*, 1915, XX, 61-72.
8. Van Slyke, D. D., Cullen, G. E., Stillman, E.: *Proceedings of the Society for Experimental Biology and Medicine*, meeting of April, 1915. Also forthcoming publication in *Journal of Biological Chemistry*.
9. Fridericia, L. S.: *Berlin klin. Wochenschrift*, 1914, LI, 1268-1270. Cf. also *Ztsch. für klin. Medizin*, 1914, LXXX, 1-12. Instrument manufactured by Paul Altmann's Fabrik für Laboratoriumsbedarf, Berlin N. W. 6, Luisenstr. 47; and by the Emil Greiner Co., 55 Fulton St., New York.
10. Benedict, S. R.: *Journal of the American Medical Association*, October 7, 1911, p. 1193-1194.



## SOME PRODUCTS OF HYDROLYSIS OF GLIADIN, LACT-ALBUMIN, AND THE PROTEIN OF THE RICE KERNEL.<sup>1</sup>

By THOMAS B. OSBORNE, DONALD D. VAN SLYKE, CHARLES S. LEAVENWORTH, AND MARIAM VINOGRAD.

(From the Connecticut Agricultural Experiment Station, New Haven, and the Hospital of The Rockefeller Institute for Medical Research, New York.)

(Received for publication, July 12, 1915.)

To settle questions concerning the quantity of some amino-acids yielded by several proteins which form important constituents of human food we have cooperated in the following analyses in order to eliminate, as far as possible, uncertainties due to inexperience in the use of methods, and also those due to working with samples of different origin. We have also paid especial attention to the determination of lysine, in order to gain further information in respect to the differences frequently encountered between the results of the direct isolation of this amino-acid as picrate and its indirect estimation by the Van Slyke method.<sup>2</sup>

### *The Amount of Lysine in Gliadin.*

The discovery of a small quantity of lysine among the products of hydrolysis of gliadin has some important bearings. In the first place it raises the question of the individuality of gliadin, for the amount of lysine isolated was so small that it seemed likely that it was derived from some contaminating protein. A rigid fractionation of 500 grams of this preparation of gliadin yielded extreme fractions from which similar small quantities of lysine were obtained, thus giving no evidence of the presence of any other protein.<sup>3</sup>

<sup>1</sup> A portion of the expenses of this investigation was also shared by the Carnegie Institution of Washington, D. C.

<sup>2</sup> Compare Van Slyke, D. D., *Jour. Biol. Chem.*, 1913-14, xvi, 531.

<sup>3</sup> Osborne, T. B., and Leavenworth, C. S., *Jour. Biol. Chem.*, 1913, xiv, 481.

That gliadin is in fact a definite substance and not a mixture of two or more proteins has recently been asserted by Gróh and Friedl<sup>4</sup> who have compared several of the physical properties of successive fractions of carefully purified gliadin, and conclude that there is only one alcohol-soluble protein in wheat gluten.

In the second place the results of an analysis by Van Slyke,<sup>5</sup> made by his indirect method, indicated a much larger quantity of lysine than that isolated as picrate, and consequently raised the question of the limits of accuracy of the determinations of lysine made by this method, as well as those by Kossel's method. The great importance of gliadin as a constituent of human food, as well as its extensive use in nutrition experiments, from the results of which conclusions frequently have been drawn on the assumption that it yields no lysine, makes it important to know, as accurately as possible, its true content of the lysine complex, as well as of its other constituents. We have therefore made four new analyses by the Van Slyke method, the closely agreeing results of which, corrected for the solubility of the bases, can now be compared with those obtained by other methods.

*Gliadin. Van Slyke Method.*

	Per cent of total nitrogen. Uncorrected.				Corrected for solubility of the bases,				Average of corrected results.
	I*	II*	III**	IV**	I	II	III	IV	
Ammonia N.....	24.27	24.68	24.67	24.83					24.61
Humin N.....	0.83	0.51	0.50	0.48					0.58
Cystine N.....	0.28	0.30	0.38	0.38	0.70	0.72	0.90	0.90	0.80
Arginine N.....	5.11	4.96	4.65	4.79	5.62	5.47	5.29	5.43	5.45
Histidine N.....	2.84	2.93	2.43	2.57	3.44	3.59	3.19	3.33	3.39
Lysine N.....	1.14	1.28	1.26	1.30	1.22	1.36	1.36	1.40	1.33
Amino N of filtrate...			52.70	53.10			51.65	52.25	51.95
Non-amino N of filtrate.....			12.07	11.08			11.10	10.30	10.70
Total.....			98.66	98.53					98.81

\* Total nitrogen = 0.624 gm.

\*\* Total nitrogen = 0.501 gm.

<sup>4</sup> Gróh, J., and Friedl, G., *Biochem. Ztschr.*, 1914, lxvi, 154.

<sup>5</sup> Van Slyke, *Jour. Biol. Chem.*, 1911-12, x, 15.

Determinations of the basic amino-acids in this same preparation of gliadin, and also in two samples obtained from it by fractional precipitation from alcohol, gave the following results:<sup>6</sup>

*Gliadin. Former Results by Kossel's Method.*

Grams from 100 gm. of gliadin.

	Arginine.	Histidine.	Lysine.
This sample I. ....	2.67	1.63	0.16
II. ....	2.78	1.49	
Fraction A I. ....	2.48	1.43	0.15
II. ....	2.43	1.44	
Fraction B I. ....	2.92	1.49	0.07
II. ....	2.90	1.48	

An earlier determination of arginine in the gliadin hydrolyzed by Osborne and Clapp<sup>7</sup> gave 3.16 per cent. Kossel and Kutscher<sup>8</sup> found in three different fractions of the alcohol-soluble protein of wheat 3.05, 2.75, and 3.13 per cent of arginine and 1.20 and 1.53 per cent of histidine.

These determinations agree as closely as can be expected when the difficulties involved in the execution of Kossel's complicated method are taken into consideration. The following figures facilitate a comparison of the average of the above results with those obtained by the Van Slyke method.

	Grams amino-acid from 100 gm. of gliadin by the methods of:	
	Kossel Former analyses.	Van Slyke.* Present analyses
Arginine .....	2.70	2.97
Histidine .....	1.49	2.19
Lysine .....	0.13	1.21

\* Calculated for 17.5 per cent of nitrogen in the gliadin.

That the figures for arginine and histidine by the Van Slyke method are somewhat higher is to be expected, for slight losses in carrying out the Kossel method are almost inevitable. We can consider, therefore, that the proportions of these two amino-acids are now fixed within reasonably satisfactory limits. The difference among the several results for lysine is relatively great, and we have therefore subjected it to further investigation.

Since the amount of lysine picrate previously obtained from this

<sup>6</sup> Osborne and Leavenworth, *loc. cit.*

<sup>7</sup> Osborne, T. B., and Clapp, S. H., *Am. Jour. Physiol.*, 1906-07, xvii, 231.

<sup>8</sup> Kossel, A., and Kutscher, F., *Ztschr. f. physiol. Chem.*, 1900-01, xxxi, 165.

sample of gliadin was lower than the amount corresponding to either Van Slyke's lysine nitrogen or the free amino nitrogen (see latter part of this paper), new determinations were made of the basic nitrogen precipitated by phosphotungstic acid according to the modified Hausmann method, and also of lysine by Kossel's method. The new direct determination of lysine was made as nearly as possible under the conditions of Hausmann's method for determining basic nitrogen, with the modification that losses which might occur on decomposing the phosphotungstate precipitate were avoided by using the amyl alcohol-ether method.<sup>9</sup>

100 gm. of the air dry gliadin, equal to 95.28 gm. ash- and moisture-free, were boiled with 1000 cc. of 20 per cent hydrochloric acid for twenty-four hours, the solution was concentrated under diminished pressure until most of the hydrochloric acid was removed, and boiled with an excess of magnesium oxide until free from ammonia. The solution was then filtered and concentrated to 1 liter. Two portions of 10 cc. each were diluted to 100 cc. and the nitrogen precipitable by phosphotungstic acid was determined by the modified Hausmann method. We thus found in each portion 0.0096 gm. of basic nitrogen, equal to 1 per cent of the gliadin, or exactly the same amount as in the determinations previously made with 1 gm. portions.

The remainder of the solution was diluted to 10 liters, 300 cc. of concentrated sulphuric acid, and then 3000 cc. of a 20 per cent phosphotungstic acid solution, containing 5 per cent of sulphuric acid, were added. After standing over night the precipitate was filtered out and washed with a 5 per cent solution of phosphotungstic acid. The precipitate was then suspended in about 500 cc. of water, to which about 10 cc. of 30 per cent sulphuric acid were added, and shaken out with a liberal quantity of a mixture of equal parts of amyl alcohol and ether. A clear solution of phosphotungstic acid in the organic solvent formed at once, the decomposition being effected with great ease. The aqueous solution was drawn off and the amyl alcohol-ether layer was washed three times with distilled water. The aqueous solution was then shaken three times more with fresh amyl alcohol-ether mixture, and the latter was each time washed with water to avoid loss.

The solution of the bases was brought to a volume of 1 liter, and nitrogen was determined in two portions of 10 cc. each, 0.86 and 0.86 mg. being found. This is equal to 0.88 gm., calculated back to the whole of the original solution which had previously been found to contain 0.96 gm. of nitrogen precipitable with phosphotungstic acid. The loss by decomposition of the precipitate was 0.08 gm., or 8.3 per cent of the basic nitrogen. How this loss occurred is not clear.

Arginine and histidine were removed from this solution by baryta and silver nitrate, as in the Kossel method, and the lysine by precipitating with phosphotungstic acid. The lysine phosphotungstate was decomposed by the amyl alcohol-

<sup>9</sup> Van Slyke, *Jour. Biol. Chem.*, 1915, xxii, 281.

ether process as just described. After removing the sulphuric acid with an excess of baryta and the latter by carbonic acid, the solution of the lysine carbonate was made up to 1000 cc. and nitrogen determined in two portions of 25 cc. each, 2.6 and 2.4 mg. being found, equal to 0.1000 gm. of nitrogen, or 0.5210 gm. of lysine, in the whole solution. Correcting this for the solubility of lysine phosphotungstate in the 15 liters from which it was precipitated gives 0.7085 gm. of lysine, equal to 0.76 per cent of the gliadin. After evaporating to a syrup and diluting with a little alcohol, an alcoholic solution containing just enough picric acid to convert this quantity of lysine into the picrate was added. A characteristic yellow precipitate formed at once, which was filtered off the next day, and when dried weighed 0.9746 gm., equal to 0.3794 gm. of lysine.

Since lysine phosphotungstate is soluble to the extent of 12.5 mg. of lysine per liter, and the volume of the solutions from which this had been precipitated was 15 liters, we must add 0.1875 gm. to the lysine found, making the total 0.5669 gm., equal to 0.64 per cent of the ash- and moisture-free gliadin. The purity of the substance weighed was shown by recrystallizing from water whereby 0.8390 gm. of pure, crystallized lysine picrate was recovered. Adding to this 0.0756 gm. for the solubility of lysine picrate in the 14 cc. of filtrate gives a total of 0.9146 gm. equal to 94 per cent of the substance weighed. The nitrogen in the solution from which the 0.9746 gm. of lysine picrate had been obtained was equivalent to 0.5210 gm. of lysine, equal to 0.1416 gm. more lysine than was isolated as picrate. The filtrate from the 0.9746 gm. of substance was evaporated to dryness, during which process a very small quantity of yellow substance, similar in appearance to lysine picrate, separated. The residue was extracted with alcohol, and the insoluble matter filtered out, washed with alcohol, and dissolved in water. The solution when evaporated to dryness left a residue which weighed only 6 mg. The alcoholic solution was also evaporated, and left a residue weighing 0.3450 gm. Since 0.7764 gm. of picric acid had been added, of which 0.5952 gm. separated with the lysine picrate, 0.1812 gm. of picric acid was therefore left in this filtrate. Deducting this from the weight of the residue left on evaporating the filtrate we have 0.1638 gm. of substance. The solution from which the lysine had been precipitated contained 0.1000 gm. of nitrogen, and the precipitated lysine 0.0728 gm., leaving 0.0272 gm. of nitrogen, equal to 16.4 per cent of the residue, providing there was no loss on decomposing the second phosphotungstate precipitate. If all of this residue was lysine it would be equal to only 0.17 per cent of the gliadin which shows that the loss of lysine in precipitating as picrate was, at the most, small.

The amount of lysine which we have thus found, 0.64 per cent, is much more than the 0.16 per cent obtained in the earlier analyses, but considerably less than the 1.21 per cent calculated from the partition of nitrogen in the Van Slyke analysis.

Since the conditions of precipitation of the phosphotungstates in the Hausmann method are quite different from those in the Van Slyke, we repeated the determination of lysine in gliadin, under the

conditions of the latter method, in order to determine whether or not the differences here encountered may thus be explained.

100 gm. of the same sample of gliadin, equal to 93.38 gm. ash- and moisture-free, were hydrolyzed as before. After removing the ammonia 400 cc. of concentrated hydrochloric acid were added and the solution was made up to 4 liters. After heating to 95°, a boiling solution of 320 gm. of phosphotungstic acid, dissolved in 1350 cc. of water was added, and the solution allowed to stand for forty-eight hours, during which time the phosphotungstates crystallized out. The precipitate was sucked out and washed with an ice cold 2.5 per cent solution of phosphotungstic acid in 0.8 normal hydrochloric acid. The precipitate was decomposed by the amyl alcohol-ether method. The sulphuric acid used in the decomposition was removed with baryta, and the arginine and histidine were removed with silver nitrate and baryta from a volume of 400 cc. Barium and silver were removed by sulphuric acid and hydrogen sulphide, and the solutions made up to 1000 cc. after adding 30 cc. of concentrated sulphuric acid.

The lysine was then precipitated from the solution heated to 95° by adding a boiling solution of 40 gm. of phosphotungstic acid dissolved in 200 cc. of 5 per cent, by volume, sulphuric acid. The lysine phosphotungstate was decomposed with baryta in the usual way and, when freed from baryta with carbonic acid, the solution was made up to 1000 cc. and nitrogen determined in two portions of 25 cc., 0.0038 gm. being found in each, equal to 0.1520 gm. nitrogen or 0.7920 gm. lysine in the entire solution. After concentrating the remaining 950 cc. of solution to a thin syrup, alcohol was added until a turbidity formed and then an alcoholic solution containing 1.18 gm. of picric acid. After standing over night the lysine picrate was filtered out, washed with alcohol, and dried at 100°. It weighed 1.23 gm., equal to 0.4788 gm. lysine. Since the first phosphotungstic acid precipitate was thrown down from 5350 cc. and the second from 1200 cc., this figure, when corrected for the solubility of lysine phosphotungstate, namely 0.0125 gm. lysine per liter, is equivalent to 0.5607 gm. of lysine in the original solution, or 0.63 per cent of the gliadin.

This agrees exactly with the result obtained by precipitating the phosphotungstates under the conditions of Hausmann's modified method, namely 0.64 per cent. It also shows that no greater loss occurred on decomposing the lysine phosphotungstate with baryta than with amyl alcohol and ether.

The filtrate from the lysine picrate was evaporated and the residue extracted with alcohol to remove excess of picric acid. The substance insoluble in alcohol was dissolved in water and every effort made to isolate lysine picrate from it, but none could be obtained. We have thus shown that gliadin contains at least 0.64 per cent of lysine and have reduced the difference between the amount found by direct determination as picrate by the Kossel method and that in-

directly to 0.57 per cent. Since the results obtained by the Kossel method are unquestionably somewhat too low and those by the Van Slyke method may be somewhat too high this difference between the two methods is probably no greater than we should expect. The amount of lysine actually yielded by this protein is probably not far from the average of these two results, namely, 0.92 per cent of the gliadin.

### *The Amide Nitrogen of Gliadin.*

The closely agreeing figures for nitrogen as ammonia in the Van Slyke analyses are of interest inasmuch as they confirm the already large number of determinations previously made on this, as well as many other preparations. In a paper<sup>10</sup> published some time ago attention was directed to the fact that proteins which yield much nitrogen as ammonia also yield much glutaminic acid, and it was suggested that this nitrogen might be in amide union with one of the carboxyl groups of the glutaminic and aspartic acids. The amount of ammonia required for such a union was calculated, and for most proteins found to correspond closely with that determined by analysis. Among the few for which a notable difference was found was gliadin. New determinations have shown a higher yield of glutaminic acid<sup>11</sup> which, together with the aspartic acid, corresponds closely with the quantity required for this assumed union with the ammonia actually yielded by gliadin, namely 5.12 per cent.

If, as is probable,<sup>12</sup> the amount of aspartic acid found is about one-half of that present, the calculated amount of ammonia, if this were in amide union, would be 5.18 per cent. The agreement between the results of analysis (5.12 per cent) and this calculation is striking, and suggests that the amount of glutaminic acid which has been obtained from glutenin, hordein, and zein may likewise be less than that actually yielded by these proteins.

<sup>10</sup> Osborne, T. B., Leavenworth, C. S., and Brautlecht, C. A., *Am. Jour. Physiol.*, 1908-09, xxiii, 180.

<sup>11</sup> Osborne, T. B., and Guest, H. H., *Jour. Biol. Chem.*, 1911, ix, 425.

<sup>12</sup> Compare Osborne, T. B., and Jones, D. B., *Am. Jour. Physiol.*, 1910, xxvi, 305.

*The Amount of Lysine in Lactalbumin.*

The only analysis of the products of hydrolysis of this protein is that published by Abderhalden and Pribram.<sup>13</sup> Since the feeding experiments with lactalbumin by Osborne and Mendel<sup>14</sup> have shown that a much smaller quantity of this protein supplements the nutritive deficiencies of the lysine-free zein than does any one of numerous other proteins, it seemed probable that it would be found to yield relatively large amounts of this amino-acid. This expectation has been realized, as the following figures show.

The preparation of lactalbumin employed for these analyses was a part of a large quantity that was used for the feeding experiments just referred to, and was made in the following manner: Nearly all the fat was separated from fresh cow's milk by centrifugation, and the casein was removed by adding very dilute hydrochloric acid until the precipitation was complete. Most of the casein was

*Lactalbumin. Van Slyke Analysis.*

Per cent of total nitrogen.

	Uncorrected.			Corrected for solubility of bases,***			Average of corrected results.
	I*	II**	III**	I	II	III	
Ammonia N . . . . .	8.26	8.84	8.60				
Humin N absorbed by lime . . . . .	2.11	2.15	2.69				
Humin N in amyl alcohol extract . . . . .		0.47	0.51				
Cystine N . . . . .	0.56	0.40	0.30	1.39	1.31	1.21	1.30
Arginine N . . . . .	6.61	6.23	6.20	7.35	7.14	7.11	7.20
Histidine N . . . . .	3.74	3.47	2.94	4.65	4.80	4.27	4.57
Lysine N . . . . .	12.19	11.91	12.17	12.27	12.09	12.35	12.24
Amino N of filtrate . . . . .	63.3	62.8	62.3	62.1	61.0	61.5	62.0
Non-amino N of filtrate . . . . .	3.65	3.5	3.3	2.5	1.8	1.6	2.0
Total . . . . .	100.45	99.77	98.91				

\* Total nitrogen = 0.556 gm.

\*\* Total nitrogen = 0.571 gm.

\*\*\* The corrections for II and III are double the usual amounts, because the bases were recrystallized from a volume of solution equal to that in which they were precipitated.

strained out on cheese-cloth and the solution then filtered through paper pulp. A perfectly clear filtrate with only a faint trace of opalescence was thus obtained. The lactalbumin was coagulated by heating rapidly to boiling, and at once filtered out and freed from chlorides and lactose by grinding with water in a "Nixta-

<sup>13</sup> Abderhalden, E., and Pribram, H., *Ztschr. f. physiol. Chem.*, 1907, li, 409.

<sup>14</sup> Osborne, T. B., and Mendel, L. B., *Jour. Biol. Chem.*, 1914, xviii, 1.



mal" mill, and then washing with boiling water. The coagulum was next digested six times with strong alcohol, whereby all but an insignificant amount of substance soluble therein was removed. This preparation, when dried in the air at room temperature, weighed 1775 gm. and contained 8.17 per cent moisture, 0.66 per cent ash, and, calculated ash- and moisture-free, 15.51, 15.47, and 15.49 per cent of nitrogen (Kjeldahl).

In regard to the chemical individuality of this preparation we can only say that it represents a mixture of all of the coagulable proteins present in the milk serum after removing casein. No attempt was made to separate these from one another as we wished to know the amino-acid make-up of the coagulum thus prepared for use in feeding experiments.

The analysis by the Van Slyke method gave the above results.

Determination I was made exactly as indicated in the original description of the method, except for the improvements in manipulation outlined in the next succeeding article. Since the result of this determination for lysine was higher than that of the Kossel determination, it was thought that some mono-amino-acids might have been occluded in the phosphotungstate precipitate of the bases, and failed to be entirely dislodged by the washing. Consequently in Determinations II and III the phosphotungstates, after being washed in the usual manner, were purified by recrystallization.

For this they were suspended in water, and dissolved by the addition of just sufficient sodium hydrate. The solution was then acidified, heated, and the bases were reprecipitated by addition of 15 cc. of concentrated hydrochloric acid and 5 gm. of phosphotungstic acid in each determination, the volume of the final mixture being 200 cc., as in the case of the first precipitation. The recrystallized precipitate of the bases was finally washed seven times with suction, and decomposed with amyl alcohol, ether, and acid, as described in the following paper. All the coloring matter which accompanied the bases was extracted by the amyl alcohol and ether. The nitrogen taken up by the organic solvents was determined by driving off the latter with vacuum distillation, taking the residue up in water, and treating by the Kjeldahl method. This is given in the table as "humins N in amyl alcohol extract."

That when the bases are thoroughly washed recrystallization is unnecessary is indicated by the fact that this process did not affect the figure for lysine, and that the amount of nitrogen and amino nitrogen in the mother liquors from the recrystallization corresponded only to the amount calculated from the solubility of the

bases. The total nitrogen in the filtrate and washings from the second precipitations in Determinations II and III was, in each case 0.0087 gram, the amino nitrogen 0.0036 gram. These amounts are added to the total figures for the filtrate, uncorrected, but deducted in the corrected figures.

The good agreement of Analysis I with II and III indicates that the phosphotungstate precipitate in all three analyses was quite pure. Otherwise the recrystallization used in II and III would have changed its composition.

In order to obtain further evidence as to the purity of the phosphotungstate precipitate, *i.e.*, whether it contained only lysine, arginine, histidine, and cystine, we have endeavored to exclude possible contamination by tryptophane, the only known amino-acid which, under the conditions of the analysis, could possibly be precipitated with the bases.

In the original paper on the hydrolysis method it was shown that tryptophane when boiled with hydrochloric acid yields a product which, in not too dilute solution, can be partially precipitated by phosphotungstic acid. The solubility of this phosphotungstate is, however, so much greater than that of the hexone base phosphotungstates, that tryptophane in order to contaminate the latter would have to be present in larger amounts than it occurs in any protein we have previously analyzed (the non-amino nitrogen of the mono-amino fraction in no case exceeded the minimum proline + oxyproline nitrogen sufficiently to make possible the presence of more than a few per cent of tryptophane). An indication of the amount of tryptophane which would have to be present in lactalbumin in order to contaminate the base precipitate is indicated by the following experiment.

0.200 gm. of tryptophane, equivalent to 5.5 per cent of the lactalbumin hydrolyzed in Analyses II and III, was boiled twenty-four hours with 50 cc. of 20 per cent hydrochloric acid. The solution was concentrated on the water bath and then treated like the hydrolysate in the Van Slyke protein analysis. Relatively little ammonia and humin nitrogen (0.6 and 1.0 mg.) was found. The filtrate from the humin was brought to 50 cc., with the addition of 2 cc. of concentrated hydrochloric acid and 4 gm. of phosphotungstic acid, the concentrations of these acids being those ordinarily used in precipitating the bases. When the solution stood over night a considerable amount of crystalline phos-

phosphotungstate separated, as in the former experiments of Van Slyke and of Gortner, where 38.7 per cent of the nitrogen was precipitated.

That the precipitation is dependent on the concentration of the tryptophane solution, however, is shown by the following:

150 cc. of a solution containing 3.5 per cent of HCl and 2.5 per cent of phosphotungstic acid were added to the above mixture after the phosphotungstate had crystallized, and the crystals were dissolved by warming. The solution was then allowed to stand several days, the dilution being that usually used in the Van Slyke protein hydrolysis. A perceptible precipitate formed, but it was slight, and contained only 1.8 mg. of nitrogen.

If this amount of tryptophane nitrogen had been present in the precipitate of the lactalbumin bases only one-fourth or 0.45 mg. of nitrogen, equivalent to 0.08 per cent of the total, would have been calculated as lysine, the base on which the Kossel and Van Slyke hydrolyses gave the most widely deviating figures, namely, 10.5 and 12.2 per cent of the total nitrogen. As the precipitate from the lactalbumin was recrystallized at equal dilution, however, at least twice as much tryptophane, or an amount equal to 11 per cent of the lactalbumin used, could have been present in the protein without appreciably affecting the lysine figures.

In order to ascertain whether there can be more than this amount of tryptophane in lactalbumin we have utilized Gortner and Blish's<sup>15</sup> discovery that 86 per cent of tryptophane can be converted into humin by adding glucose to the hydrochloric acid solution in which the protein is hydrolyzed. The following experiment confirms Gortner on this point.

0.200 gm. of tryptophane and 1 gm. of glucose were boiled twenty-four hours with 50 cc. of 20 per cent hydrochloric acid. The solution, unlike a tryptophane-HCl solution without glucose, blackened during the boiling. Thereafter it was treated like the hydrolysate in the Van Slyke protein analysis, with the following results.

	N mg.	Per cent of the N.
Ammonia .....	0.69	2.5
Humin .....	23.74	86.5
Phosphotungstate precipitate .....	1.16*	4.2
Filtrate (by difference) .....	1.87	6.8
	<u>27.46</u>	

\* This precipitate was brown, flocculent, of low specific gravity. It appeared more like humin than that had escaped precipitation with calcium hydrate than like the amino-acid phosphotungstates, which are crystalline and heavy.

<sup>15</sup> Gortner, R. A., and Blish, M. J., *Jour. Am. Chem. Soc.*, 1915, xxxvii, 1630.

In the lactalbumin analysis 7 grams of albumin and 7 grams of glucose were boiled with 200 cc. of 20 per cent hydrochloric acid. The hydrolysis was continued forty-six hours, as a preliminary experiment indicated that the glucose retards somewhat the rate of hydrolysis. The hydrolyzed solution was brought to 250 cc., and 100 cc. containing 0.3948 gram of nitrogen were taken for analysis.

*Lactalbumin IV. Hydrolyzed in the Presence of Glucose.*

	Uncorrected.	Per cent of total nitrogen,* Corrected for solubility of the bases.
Ammonia .....	8.37	
Humin .....	3.70	
Cystine .....	0.39	1.05
Arginine .....	7.29	8.10
Histidine .....	2.26	3.22
Lysine .....	12.42	12.54
Amino N of filtrate .....	60.10	58.79
Non-amino N of filtrate ? .....	4.82	3.58
Total .....	99.35	

\* Total nitrogen = 0.395 gm.

The chief effect of the glucose is to increase the humin nitrogen from 2.1–2.7 up to 3.7 per cent. The total nitrogen of the bases is not reduced at all, the percentage, corrected for solubility, being 25.66 with glucose, compared with 24.94 to 25.91 without glucose. Nor are the proportions of amino to non-amino nitrogen in the bases significantly affected. (The lower histidine figure in Analysis IV is due to the high result in the arginine determination, not to differences in either the total, or the amino N of the bases.)

The above results show in two ways that tryptophane could not have interfered with the figures for the bases.

1. There was not enough tryptophane present. Making the apparently justified assumption that the 3.7 per cent of humin nitrogen represented 86 per cent of the tryptophane nitrogen, the maximum amount of tryptophane nitrogen present would be 4.3 per cent of the total, equivalent to 4.85 per cent of tryptophane in the protein. The experiment with tryptophane reported on a preceding page indicates that over twice as much would have had to be present in order to contaminate the bases appreciably.

2. Removing the tryptophane by changing it into humin did not affect the figures for the bases.

The distribution of nitrogen according to the modified Hausmann<sup>16</sup> method was determined with the following results.

*Hausmann Analysis of Lactalbumin.*

	Per cent of the lactalbumin.		
	I.	II.	Average.
Ammonia N .....	1.31	1.33	1.32
Humin N .....	0.55	0.53	0.54
Basic N .....	3.62	3.59	3.61
Non-basic N .....	10.01	10.04	10.02
Total N .....	15.49		

If we compare the corresponding data from the Van Slyke hydrolysis we obtain figures which agree well:

	Per cent of the lactalbumin.	
	Van Slyke.	Hausmann.
Ammonia N .....	1.31	1.32
Humin N .....	0.36	0.54
Basic N .....	3.44	3.61
Non-basic N .....	10.38	10.02

Comparing the percentages of these bases, as determined directly by the Kossel method, with those calculated from the results of the Van Slyke method we have in per cent of the lactalbumin:

	Per cent of the lactalbumin.		
	I. Kossel.	II. Van Slyke.	
Arginine .....	3.01	3.00	3.47
Histidine .....	1.53	1.52	2.61
Lysine .....	8.06	8.10	9.89

The agreement between these analyses for arginine is satisfactory, but for histidine and lysine the difference is too great.

Since for the hydrolysis by the Kossel method sulphuric acid was used, it appeared possible that the decomposition of the lactalbumin might not be as complete as with hydrochloric acid, which was used for the Van Slyke analysis. Furthermore, as suggested by Van Slyke,<sup>17</sup> losses also might have been caused by adsorption during the process of decomposing the voluminous phosphotungstate pre-

<sup>16</sup> Osborne, T. B., and Harris, I. F., *Jour. Am. Chem. Soc.*, 1903, xxv, 323.

<sup>17</sup> Van Slyke, *Jour. Biol. Chem.*, 1913-14, xvi, 531.

precipitates with baryta. We therefore made new determinations of lysine, making the precipitations with phosphotungstic acid as nearly as possible under the conditions of the Van Slyke method. Although the yield of lysine was increased to 8.8 per cent, calculated on the unrecrystallized picrate, or 8.45 per cent, calculated from the recrystallized product the figure could not be raised to the level (9.9 per cent) estimated by the Van Slyke method.

In making this analysis 50 gm. of the same preparation of the air dry lactalbumin, equal to 45.45 gm. ash- and moisture-free, were boiled with hydrochloric acid for twenty-four hours, and ammonia was removed by heating with magnesia. The solution was then divided into equal parts, the second phosphotungstate precipitate of the lysine from Part I being decomposed with baryta, and that from Part II by shaking with dilute sulphuric acid and a mixture of amyl alcohol and ether.

The final solutions containing the lysine sulphate were made up to 1000 cc. each. In two portions of 25 cc. of Solution I we found 0.0102 and 0.0100 gm. nitrogen, and of Solution II 0.0102 and 0.0102 gm., equal to 0.4080 gm. in each of the total solutions. These results show that in this case, at least, there was no loss of lysine through adsorption by the voluminous barium phosphotungstate. Since the phosphotungstates were precipitated from a volume of 2642 cc., the nitrogen found should be increased by 0.0066 gm. on account of the solubility of lysine phosphotungstate, equal to 0.0025 gm. nitrogen per liter, which makes the corrected lysine nitrogen 0.4146 gm., equal to 2.1393 gm. of lysine, or 9.60 per cent of the lactalbumin, as against 9.89 per cent estimated by the Van Slyke method.

To determine how much of this nitrogen could be isolated as lysine picrate the remaining 950 cc. of Solutions I and II were treated with picric acid and the precipitates dried at 100°. These weighed 4.71 and 4.69 gm., equivalent to 8.82 and 8.78 per cent of lysine respectively, after correcting for solubility of the phosphotungstate precipitates and for the portions previously removed for nitrogen estimations. The precipitated lysine picrate was recrystallized from water and 4.44 and 4.41 gm. were obtained. Adding 0.0810 gm. for the solubility of the picrate in the 15 cc. of mother liquor gives 4.52 and 4.49 gm., equal to 95 per cent of the precipitated lysine picrate recovered analytically pure.

The corrected percentage of the recrystallized picrate, 8.47 and 8.42, is comparable with that obtained in the earlier analyses, namely, 8.06 and 8.10. This slightly higher result may be due to a more complete hydrolysis by hydrochloric acid than by sulphuric acid, or to a more complete decomposition of the first phosphotungstic precipitate of the arginine, histidine, and lysine by the amyl alcohol-ether method, compared with the baryta method. The latter assumption is improbable, however, because the second precipitate of

lysine phosphotungstate, although decomposed with baryta, yielded as much lysine as the first.

We have thus been able to isolate lysine picrate in a pure crystalline condition equal to 8.47 per cent or to 8.82 per cent in the form of the amorphous precipitate, which was at least 95 per cent pure and probably much purer. The solution from which this lysine picrate was obtained contained nitrogen equal to 9.6 per cent of lysine.

Since a very careful examination of the filtrates from the lysine picrate failed to yield even traces of this salt, the picric acid was removed by acidifying the concentrated solutions with hydrochloric acid, filtering out the greater part of the picric acid which crystallized out, and removing the remainder by shaking with ether. To the suitably concentrated solutions phosphotungstic acid was added, whereupon relatively considerable precipitates formed at once. These were decomposed by hydrochloric acid and amyl alcohol and ether, and the solution was evaporated to remove excess of acid. From one of these solutions an attempt was made to obtain more lysine picrate, but none whatever could be secured. In view of the not inconsiderable amount of nitrogen unaccounted for, it seems highly probable that lysine picrate could have been thus obtained had this nitrogen belonged to lysine.

The other solution was freed from chlorine with silver carbonate, silver was removed from the filtrate from the silver chloride by hydrogen sulphide, and the solution was evaporated to small volume. This was very strongly alkaline to litmus, became turbid on adding alcohol, and after standing, yielded a crystalline precipitate. Owing to the small amount of the substance contained in this solution it was impossible to determine its nature.

It is evident that besides lysine these solutions contained some other substance or substances precipitated with the lysine phosphotungstate, and that this fact may explain in part the higher result for lysine in lactalbumin given by the Van Slyke method. On the other hand, the amount of cystine which was precipitated by phosphotungstic acid in the Van Slyke analysis, and therefore would be expected in the lysine fraction here, would account for all of this undetermined nitrogen, and leave the deficit to be explained only by the unavoidable losses of manipulation.

### *The Protein of Polished Rice.*

Six hundred grams of commercial polished rice were washed with water and then allowed to soak over night at room temperature. The softened grains were next ground to a thin paste in a special

mill, together with 2 liters of water, which included that in which they had been soaked. Two liters of 0.2 per cent sodium hydroxide solution were added, the insoluble matter was allowed to settle, the solution syphoned off, and the residue centrifuged. The entire extract thus obtained was filtered perfectly clear through a pulp filter and precipitated with dilute acetic acid, added until the protein separated sharply. After the precipitate had settled the solution was drawn off and replaced by alcohol equal to three times the volume of the solution which remained. This was done to remove any alcohol-soluble protein that might be present, and which could best be extracted from the finely divided precipitate before it was rendered more compact by collecting on a filter. After standing in this dilute alcohol for two days the precipitate was boiled out twice with 500 cc. of 80 per cent, by volume, alcohol, and dehydrated with absolute alcohol. The air dry preparation weighed 25 grams, equal to 4.17 per cent of the polished rice, and contained 7.98 per cent moisture, 0.78 per cent ash, and 15.20 and 15.29 per cent nitrogen, equal to 16.68 per cent calculated for the ash- and moisture-free substance.

The alcoholic extracts were concentrated and found to contain a very little protein, similar to gliadin from wheat in its solubility. The amount was too little to enable us to learn anything about it, and we were also unable to determine whether it was an original constituent of the seed.

*Rice Protein.*

	Per cent of total nitrogen.*				Grams amino-acid per 100 gm. of protein.**
	I.	II.	Average.	Average corrected for solubility of bases.	
Ammonia N.....	11.23	11.43	11.33		
Humin N.....	1.46	1.72	1.59		
Cystine N.....	0.41	0.50	0.46	0.88	1.26
Arginine N.....	16.85	17.50	17.17	17.69	9.15
Histidine N.....	4.54	4.99	4.77	5.39	3.32
Lysine N.....	4.81	4.82	4.82	4.90	4.26
Amino N of filtrate.....	52.95	53.00	52.98	52.13	
Non-amino N of filtrate.....	6.05	6.10	6.08	5.28	
Total.....	98.30	100.16			

\* Total nitrogen = 0.611 gm.

\*\* Calculated on the assumption that the ash-free protein contains 16.68 per cent of nitrogen.



The preparation of the rice protein, which is equal in amount to somewhat more than one-half of the protein of the polished rice, represents the glutelin of the seed which Rosenheim and Kajiura have named oryzenin.<sup>18</sup> This protein has been hydrolyzed by Suzuki, Yoshimura, and Fuji<sup>19</sup> who identified arginine, histidine, and lysine among its decomposition products, but failed to make satisfactory quantitative determinations of these amino-acids. We therefore thought it would be of interest to analyze our preparation by the Van Slyke method, and have done so with the preceding results.

In the following table the amount of basic substances yielded by oryzenin, which probably constitutes nearly all of the protein of the endosperm of rice, is compared with that yielded by the proteins of the endosperm of the seeds of wheat and maize. The figures for wheat are calculated on the assumption that wheat gluten, which contains nearly all of the protein of the endosperm of the seed, consists of equal parts of gliadin and glutenin; those for maize on the assumption that corn gluten, which represents the protein of the endosperm of the maize kernel, consists of two parts of zein and one of maize glutelin.

*Basic Substances Yielded by the Proteins of the Endosperm of the Seeds in Per Cent of the Protein.*

	Wheat.	Maize.	Rice.
Arginine .....	3.86	3.39	9.15
Histidine .....	2.02	1.55	3.32
Lysine .....	1.58	0.97	4.26
Ammonia .....	4.62	3.13	2.22
Percentage of assimilable nitrogen* .....	40	29	88

\* The figures for assimilable nitrogen are taken from the work done in Rubner's laboratory by Karl Thomas.<sup>20</sup> The different proteins were taken in moderate amounts after a period on a protein-free diet, when the body was presumably in a condition to retain and assimilate as much of the absorbed protein as the amino-acid content rendered possible. The percentage of absorbed nitrogen incorporated into, or at least retained by, the body tissues, was calculated, with the results given above. It is rather striking that the figures for the utilizability of these proteins correspond so closely with the lysine which they yield.

<sup>18</sup> Rosenheim, O., and Kajiura, S., *Jour. Physiol.*, 1907-08, xxxvi, pp. liv-lv.

<sup>19</sup> Suzuki, U., Yoshimura, K., and Fuji, S., *Jour. College of Agriculture, Tokyo Imperial University*, 1909, i, 77.

<sup>20</sup> Thomas, K., *Arch. f. Anat. u. Physiol., Physiol. Abt.*, 1909, 219.

From these figures it is evident that the protein of the rice kernel does not show the marked differences which the other cereal proteins show in respect to their amino-acid make-up when compared with the majority of food proteins, and particularly with most tissue proteins. It is, therefore, not improbable that the nutritional requirements may be satisfied by a smaller quantity of this protein than of those of wheat or maize kernels. These facts are in harmony with the extensive use of rice as food for men despite its low content in protein; and furthermore with the biological utilizability of the respective proteins as determined by Thomas.

#### *Free Amino Nitrogen of the Proteins.*

Van Slyke and Birchard have determined in a series of proteins the amount of nitrogen which reacts as free  $\text{NH}_2$  with nitrous acid.<sup>21</sup> In all the native proteins analyzed by them, except a gliadin preparation of doubtful purity, the free amino nitrogen was found equal to one-half the lysine nitrogen, as determined by Van Slyke's nitrogen distribution method. This relationship, in conjunction with the fact that the free amino nitrogen of the native proteins reacts with nitrous acid at the slower rate characteristic of the  $\omega\text{-NH}_2$  group of lysine as compared with the faster acting  $\alpha\text{-NH}_2$  groups of the other amino-acids, led to the conclusion that the  $\omega\text{-NH}_2$  group of the lysine is free in the native proteins, and is the only free amino group therein, all the  $\alpha\text{-NH}_2$  groups of the constituent amino-acids being bound in peptide linkings.

Recently Hartley<sup>22</sup> has added further evidence in support of this conclusion. He finds that in the albumin and the globulins of horse and ox serum the free  $\text{NH}_2$  is equal to half the lysine nitrogen.

In order to find whether the 2:1 ratio between lysine nitrogen and free amino nitrogen holds for lactalbumin and rice protein, and for gliadin, we have determined the free amino nitrogen in all three proteins and compared the results with those for lysine.

<sup>21</sup> Van Slyke, D. D., and Birchard, F. J., *Jour. Biol. Chem.*, 1913-14, xvi, 539. The evidence showing that nitrous acid does not hydrolyze the proteins under the conditions of the determination is given in this paper.

<sup>22</sup> Hartley, P., *Biochem. Jour.*, 1914, viii, 544.

0.75 gm. of each protein (air dried) was dissolved and diluted to 25 cc. 5 cc. portions were taken for Kjeldahl determinations, and 2 cc. for amino nitrogen by the nitrous acid method.<sup>23</sup> The solvents used were different. The gliadin was dissolved in 2.5 cc. of glacial acetic acid plus 1 cc. of water, and the clear solution diluted up to 25 cc. The lactalbumin was dissolved in 5 cc. of cold 10 per cent sodium carbonate, and the turbid solution diluted to 25 cc. The rice protein was taken up with sufficient 4 per cent potassium carbonate to make the volume 25 cc. Blanks were run in duplicate in each case with acetic acid or carbonate of similar concentration, the volume of gas obtained from the blank being subtracted from that obtained in the analysis. The results are given in the following table:

*Free Amino Nitrogen of the Proteins Compared with the Lysine Nitrogen.*

Protein.	Total N in 2 cc. solution.	N gas from 2 cc.	Pres- sure.	Tem- pera- ture.	Amino N in 2 cc. solu- tion.	Per cent of total N as am- ino N.	Per cent of total N = $\frac{1}{2}$ the lysine N as deter- mined by method of:	
							Van Slyke.	Kossel.
	mg.	cc.	mm.	°C.	mg.			
Lactalbumin.....	9.21	1.07	759	25	0.588	6.49	6.10	5.45
Average.....		1.05						
Rice protein.....	7.92	0.30	751	19	0.172	2.17	2.00	—
Average.....		0.30						
Wheat gliadin*.....	9.40	0.11	762	25	0.058	0.62	0.66	0.32
Average.....		0.105						

\* Repetition with more material gave the same result. A solution three times as concentrated gave 0.31 cc. of nitrogen gas at 26°, 760 mm., equivalent to 0.61 per cent of the total nitrogen.

It is evident from the above table that the ratio  $\frac{\text{lysine N}}{\text{free NH}_2 - \text{N}} = \frac{2}{1}$  holds for all three proteins when the lysine determinations by the Van Slyke method are taken. The lysine nitrogen figures for lactalbumin and gliadin by Kossel's method run somewhat lower, but parallel.

#### SUMMARY.

I. From gliadin an amount of analytically pure lysine picrate was isolated equivalent to a lysine content of 0.64 per cent of the ash free protein, correction for the solubility of lysine phosphotungstate being made. The amount obtained is several times that reported by

<sup>23</sup> Van Slyke, The Gasometric Determination of Aliphatic Amino Nitrogen in Minute Quantities, *Jour. Biol. Chem.*, 1913-14, xvi, 121.

previous authors (0 to 0.16 per cent), from which figures gliadin has heretofore been regarded as practically lysine-free.

The lysine content estimated from the partition of nitrogen by the Van Slyke method was still higher, 1.21 per cent.

As the results by the picrate method are based on the weight of pure substance actually isolated, they are minimum figures. The nitrogen partition results, on the other hand, may be taken as maximum figures; for they represent all the basic amino nitrogen not in the form of arginine, histidine, and cystine. It appears probable, therefore, that the true lysine content of gliadin lies between the results by the two methods, and may be stated as  $0.93 \pm 0.28$  per cent.

Expressed in the same manner, the histidine content is  $1.84 \pm 0.35$  per cent, the arginine  $2.84 \pm 0.14$  per cent, the upper limit in each case giving the results by the Van Slyke method, the lower that by Kossel's.

In the case of lysine especial efforts were made to eliminate all sources of loss which might account for the lower results by Kossel's picrate method as compared with the distribution method; but, as indicated above, without raising the results by the former method to the level of the latter. The lower results by Kossel's method were not due to hydrolysis with sulphuric acid instead of hydrochloric, or to precipitation of the phosphotungstates under conditions different from those employed in the Van Slyke method, or to loss in decomposition of the lysine phosphotungstate with baryta.

2. The amount of nitrogen yielded by the gliadin as ammonia corresponds closely with that required for amide union with one carboxyl group of the larger amount of glutaminic and aspartic acids recently obtained from gliadin. This fact is added evidence that the nitrogen yielded as ammonia by acid hydrolysis exists in the protein molecule in acid-amide form as in asparagine and glutamine

3. In all three proteins, as in the series analyzed by Van Slyke and Birchard and by Hartley, the free amino nitrogen determinable by the nitrous acid method was found equal to one-half the amount of lysine nitrogen determined by Van Slyke's method. As in all cases Kossel's picrate method gave somewhat lower, though parallel, results for lysine, the lysine determined as the picrate was less than

that calculated from the free amino nitrogen of the proteins, although the deviation was not great. The approximate constancy of the ratio, free  $\text{NH}_2$  — N : lysine N = 1 : 2, indicates that one of the two  $\text{NH}_2$  groups of lysine exists free in the protein molecule, the free group being probably the  $\omega$ - $\text{NH}_2$  group.

4. The hexone base content of lactalbumin was found to be the following, the upper limit in each case representing the results by Van Slyke's method, the lower that by Kossel's: lysine,  $9.16 \pm 0.68$  per cent; histidine,  $2.06 \pm 0.54$  per cent; arginine,  $3.23 \pm 0.23$  per cent.

As in the case of gliadin, it was found impossible to isolate an amount of lysine picrate corresponding to the entire content indicated by the nitrogen distribution method. The difference between the two sets of results could not be reduced below 1.3 per cent, despite exceptional precautions to eliminate error in both methods. Both agreed, however, in showing that lactalbumin is exceptionally rich in lysine. This fact is particularly interesting in view of the ability of lactalbumin, shown by Osborne and Mendel, to stimulate the growth of rats when used to supplement a ration low in lysine.

5. The partition of nitrogen among the products of hydrolysis of oryzenin, the chief protein of the endosperm of rice, was determined by the Van Slyke method. Compared with the endosperm proteins of wheat or maize, the protein of rice yields relatively much of each of the basic amino-acids, arginine, histidine, and lysine, and comparatively little ammonia and non-amino nitrogen. In its general amino-acid make-up it more nearly resembles the majority of the proteins of animal tissues than do the proteins of maize or wheat. This may explain the extensive use of rice as an almost exclusive diet in spite of its low protein content.

## IMPROVEMENTS IN THE METHOD FOR ANALYSIS OF PROTEINS BY DETERMINATION OF THE CHEMICAL GROUPS CHARACTERISTIC OF THE DIFFERENT AMINO-ACIDS.

By DONALD D. VAN SLYKE.

(From the Hospital of The Rockefeller Institute for Medical Research.)

(Received for publication, July 12, 1915.)

Although the principle of this method for determining the products of protein hydrolysis remains as described in the original article in this Journal,<sup>1</sup> the technique has been so modified at a number of points that the liability to error has been diminished and the ease and sureness of the manipulations increased. Brief mention of these points therefore appears justified.

In *washing the phosphatungstic precipitate of the bases* it is possible, with a steady and moderately strong suction, to dispense with the special form of plaited filter described in the original paper, and utilize a two-inch Buchner funnel with a flat circle of hardened filter paper pressed over the perforations in the usual manner.<sup>2</sup> The washing is carried out without at any time releasing the suction. Successive portions of 10 to 15 cc. of the washing solution are poured onto the well packed precipitate, which is stirred up with each portion by means of a flat-tipped rod, so that all parts of the precipitate are reached. If a steady suction is maintained all the time, this can be done without danger of loosening the filter from the floor of the funnel, and is a considerably neater and easier process than that originally described. It can not be utilized with a strong central vacuum, as this sucks the washing solution through before it can be stirred

<sup>1</sup> Van Slyke, D. D., *Jour. Biol. Chem.*, 1911-12, x, 15.

<sup>2</sup> This simplification was introduced by Dr. Percival Hartley, of the Lister Institute, during the course of his analyses of serum proteins (*Biochem. Jour.*, 1914, viii, 541).

up with the precipitate. Nor can it be used with a weak pump, as the filter paper will not be held strongly enough to the floor of the Buchner funnel to prevent its detachment during the stirring. With a good aspirator pump of the ordinary type, however, this technique is preferable to the original, because the area of filter paper that must be quantitatively washed is much smaller.

The *washing solution should be cooled to  $0^{\circ}$*  before it is poured onto the precipitate. It has been found that the solubility of the phosphotungstates of the hexone bases is only about one-fourth as great at  $0^{\circ}$  as it is at room temperature. Consequently when the washing solution is used at or near  $0^{\circ}$  the danger of dissolving appreciable amounts of the precipitate is reduced to a minimum.

The above suggests the obvious desirability of not only washing, but precipitating the bases at  $0^{\circ}$ . We can not yet say that this is a safe procedure, however. The phosphotungstates of some of the mono-amino-acids, such as alanine, phenylalanine, and proline, are fairly insoluble at  $0^{\circ}$ , and it is possible that an attempt to precipitate the bases in a solution cooled to this temperature might result in some cases in the precipitation of such acids of the mono-amino fraction.

The number of washings necessary in each case to free the phosphotungstate precipitate completely from the mother liquors is determined by *testing the successive washings* for calcium. For this a 10 per cent sodium hydrate solution containing some sodium oxalate is used. The specific gravity of this solution is so high that the drops of washing solution tested can be floated in a layer on top, and the sensitiveness of the test made much more delicate than when the portion of washing liquid to be tested is mixed with several volumes of oxalate solution. For the test, 1 or 2 cc. of the alkaline oxalate solution are placed in a test-tube of 8 or 10 mm. diameter, and 2 or 3 drops of the washings from the stem of the Buchner funnel are allowed to run down the side of the tube and form a layer on top of the solution. The tube is now gently shaken until just enough alkali has mixed with the upper layer to turn the latter alkaline. The tube is viewed against a dark background, but with plenty of light passing through the solution itself. If no cloud is visible in the upper layer, the tube is allowed to stand a few minutes.

If no evidence of a precipitate can then be seen, the washing is complete.

The fact that under these conditions the washing out of the mono-amino fraction is really complete, is shown by the results of recrystallization of the basic phosphotungstates (see lactalbumin analysis in the preceding paper, p. 342 for example). The mother liquors from the recrystallized bases were found to contain amino and non-amino nitrogen only in the amounts to be expected from the solubility of the bases. If in any analysis, however, there should be reason to doubt the purity of the phosphotungstate precipitate, or the efficiency of the washing, the precipitate may be *recrystallized* in the manner described in the report of the lactalbumin analysis.

For *decomposition of the basic phosphotungstates* (removal of the phosphotungstic acid and putting the bases into solution again) we have found quite a different procedure to be preferable, as a rule, to the use of barium originally recommended. Winterstein<sup>3</sup> showed that when a precipitate of basic phosphotungstates was suspended in dilute hydrochloric acid and shaken with ether the precipitate went into solution, the greater part of the phosphotungstic acid being extracted from the aqueous mixture by the ether. By shaking with repeated portions of ether, all the phosphotungstic acid could be removed, and concentration of the water solution yielded as a residue the pure hydrochlorides of the bases. Our experience indicated that with this method the complete removal of phosphotungstic acid, such as is necessary before the cystine sulphur can be determined, is, though possible, extremely difficult. Jacobs some time ago discovered, however, that amyl alcohol has a surprising affinity for phosphotungstic acid, and extracts it with great readiness from water solution.<sup>4</sup> We find that a mixture of equal parts of ether and amyl alcohol serves perfectly for decomposing the phosphotungstic precipitate of the hexone bases. It is preferable to amyl alcohol alone because of the lighter specific gravity of the ether mixture, and it appears to extract phosphotungstic acid from water as readily and completely as does amyl alcohol. The following procedure is utilized.

<sup>3</sup> Winterstein, E., *Ztschr. f. physiol. Chem.*, 1901-02, xxxiv, 153.

<sup>4</sup> Jacobs, W. A., *Jour. Biol. Chem.*, 1912, xii, 429.



The basic precipitate from the 3 or 4 grams of protein ordinarily used in an analysis is removed from the filter by a spatula and washing, and transferred to a half-liter separatory funnel, using 200 or 300 cc. of water to effect the transfer. Five or 10 cc. of concentrated hydrochloric acid are added, and the mixture is shaken with the 1:1 amyl alcohol-ether, using so much of the latter that it all floats in a layer above the water after the precipitate has gone into solution. Usually about 100 cc. of the ether-amyl alcohol suffice, and one or two minutes' shaking results in complete solution of the precipitate. If too little of the ether-amyl alcohol has been used, some of it will, after taking up phosphotungstic acid, sink as an oil below the aqueous phase. In case this happens, more of the extracting mixture is added, until all floats in one layer at the top.

In some cases the aqueous and ether-amyl alcohol layers do not separate readily with a clean boundary between them. This effect is due to the presence of a slight amount of humin which may have escaped previous adsorption by calcium hydrate. In this case the unadsorbed humin is carried down with the basic phosphotungstates, and fouls the solution when their precipitate is decomposed as above described. In order to clear the solution up, it is all, without separation of the aqueous and ether-amyl alcohol layers, passed through a Buchner funnel with suction. The clear filtrate readily separates into two layers. The aqueous layer is extracted with three more successive portions of ether-amyl alcohol, using each time a volume of the mixture equal to about one-fourth the volume of the water solution. Finally the combined amyl alcohol-ether extracts are shaken out once with water, to remove traces of bases that might have been carried into the extract; this portion of water is then shaken once or twice with fresh amyl alcohol-ether, and combined with the main solution of the bases. The latter should be free of phosphotungstic acid, as demonstrated by the absence of a precipitate when a few drops are added to a saturated solution of barium hydrate in a small test-tube. The solution of the bases is now concentrated to dryness in vacuum in order to drive off the free hydrochloric acid, and the residue is washed into a 50 cc. flask.

The above procedure is much quicker than the decomposition with sodium hydrate and barium chloride originally described, involves the

use of smaller volumes of solution, and finally gives a solution of the bases that contains no barium. This is of great advantage in the subsequent arginine determination, where the presence of barium is likely to cause disagreeable bumping.

The *determination of amino nitrogen in the bases* can now, with the micro-amino apparatus, be performed with only 1 or 2 cc. of the total 50 cc. It is consequently desirable to use the micro-apparatus and perform duplicate determinations of amino nitrogen, rather than use the larger amino apparatus with 10 cc. of solution, and be limited to a single determination.

The *total nitrogen of the bases*, as determined in the arginine residue, can also be determined advantageously in duplicate. The alkaline solution, left after decomposition of the arginine and removal of the ammonia formed, is diluted up to 100 or 200 cc. and divided into halves. Each half of this solution is then Kjeldahled separately, using 20 cc. of sulphuric acid for the digestion.

In conclusion it may be well to call attention again to the fact that the method was designed for use only with proteins not accompanied by other classes of substances, particularly nitrogenous substances, which would obviously falsify the interpretation of the results unless the behavior of the non-protein substances were so accurately known that corrections could be made.

Reference to the accompanying paper by Osborne, Van Slyke, Leavenworth, and Vinograd will show that with the above changes in technique the agreement between duplicate determinations is much closer than that claimed for the method in its original description.<sup>5</sup>

<sup>5</sup> *Humin nitrogen as a measure of tryptophane.* Gortner and Blish (*Jour. Am. Chem. Soc.*, 1915, xxxvii, 1630) have recently shown that if tryptophane and glucose are boiled together in 20 per cent hydrochloric acid under the conditions used for the protein hydrolysis, 86 per cent of the tryptophane is converted into humin. We have repeated the experiment with the same result. It appears highly probable that if an amount of glucose equal to that of the protein be added to the protein-acid mixture before hydrolysis, the humin nitrogen divided by 0.86 will approximately indicate the maximum amount of tryptophane. Gortner and Blish have shown that the action of glucose increases the humin yield of zein and gliadin, and we have found the same for lactalbumin. It may be well to mention that it appeared necessary to boil the lactalbumin in the presence of the glucose 48 hours, instead of 24, in order to attain complete hydrolysis.

## PROLONGED FASTING IN DIABETES.<sup>1</sup>

By FREDERICK M. ALLEN, M.D.

*(From the Hospital of The Rockefeller Institute for Medical Research.)*

Partial pancreatectomy, with preservation of the pancreatic duct so as to avoid atrophy of the remnant, gives a more satisfactory reproduction of clinical diabetes than is afforded by von Mering and Minkowski's total pancreatectomy or Sandmeyer's method of slow pancreatic atrophy. Simple lowering of the sugar tolerance without diabetes, or diabetes varying in intensity from the mildest to the very severe, can thus be produced at will. As previously pointed out,<sup>2</sup> animals thus prepared constitute valuable test-objects and afford favorable opportunities for research concerning diabetic therapy. In the milder types the measures ordinarily used in human diabetes, namely, restriction of carbohydrate or protein and brief fasting if necessary, suffice to keep the animals sugar-free and in good condition indefinitely. But in a more severe type these measures do not suffice, and the animal quickly passes into a hopeless condition if more radical treatment is delayed. In this type the initial fast must sometimes be measured in weeks rather than in days. The subsequent diet must be such as to keep the animal at a low level of weight and metabolism. Certain influences which increase either weight or metabolism tend to bring back glycosuria in these animals as in human patients. If glycosuria persists in any animal, the result is a downward sequence of lowered tolerance, emaciation, weakness, cachexia, and death, with parallel degenerative changes in the islands of Langerhans. If glycosuria is prevented, the animals may remain lively and strong though thin; they

<sup>1</sup> Read at the meeting of the Association of American Physicians, Washington, D. C., May, 1915.

<sup>2</sup> Allen, F. M., *Studies Concerning Glycosuria and Diabetes*, Harvard University Press, Cambridge, Massachusetts.

may improve somewhat with time, and the islands seem to be spared. This evidence supports the hypothesis that one set of influences overtax and injure the internal function of the pancreas, and the opposite set of influences protect and rest the internal function of the pancreas.

The treatment of diabetes at the Rockefeller Hospital has been based upon these animal experiments. The patients admitted have been forty-four, chosen as the most severe out of a considerable number of applicants, and representing a sufficient variety as respects age, social condition, and other factors. Certain points may here be mentioned concerning (1) the initial fast and (2) the subsequent diet.

Fasting has been employed by writers from Naunyn to Guelpa. The same writers describe a numerous class of diabetics who are not made sugar-free, but go on with glycosuria and acidosis and die in coma. Other patients are made sugar-free only after months of strict dietary treatment, including occasional single or repeated fast-days. Present experience indicates that the glycosuria in cases of even the severest type of diabetes may with advantage be cleared up by one initial fast. The necessary duration may sometimes be as long as eight or ten days. In one case of maximum severity in a youth, aged seventeen years, the changes accompanying fasting were studied by Dr. Eugene Du Bois in the respiration calorimeter. He found that at the outset the total metabolism was about 8 per cent. above normal and the patient was excreting all or nearly all of the sugar formed from protein and burning practically no sugar at all. In a nine days fast the glycosuria ceased, the total metabolism fell to about 20 per cent. below normal, and the respiratory quotient showed that the sugar formed from protein was being burned. It will be remembered that respiratory quotients in the most severe cases during the "oat cure" do not show increased combustion of carbohydrate. The above metabolic observations are new and constitute important evidence for the functional element in the etiology of human diabetes, since they show to what extent recovery can take place within a few days when the most complete possible rest is given the weakened metabolic function.

Severe acidosis, which is known to be diminished by the shorter

fasts previously employed, is still more reduced by the more prolonged fasting. Dangerously weak and emaciated patients have borne the fasting with apparent benefit, giving the impression that they had been suffering more from intoxication than from lack of nutrition. Alcohol is valuable during fasting as a food which does not produce glycosuria, though its use is not essential. Broadly speaking, freedom from glycosuria seems attainable in all cases of uncomplicated human diabetes before there is danger of death from starvation. In a few cases seen personally or described by others, death from some severe complication has occurred before the urine became sugar-free. In such cases to date the complication has been a severe infection, or some condition strongly tending to increase metabolism or produce nervous or circulatory disturbance. The fasting has not appeared harmful even in these few cases where it has not been successful. One case of incipient gangrene and one dangerous carbuncle cleared up rapidly under fasting, and threatening complications of infectious or any other character are considered an indication for the radical treatment as described. No contraindication has been met, unless it be the appearance of nausea, vomiting, and prostration while fasting. One woman died in such an attack; but there were attacks of this character in her previous history, and it is not positive that either the attack or the death was due to the fasting. One man began to vomit and feel unwell on the seventh day of fasting. He was fed, and the symptoms immediately passed off. After two weeks of restricted diet a second fast easily cleared up the glycosuria. This simple precaution apparently prevents danger even in this rare type of cases, and in other cases there has never been any sign of danger or harm.

Though the initial fast, to clear up glycosuria and other symptoms, is generally a very simple matter, the subsequent diet, to maintain this condition, is sometimes difficult. It is unquestionably true, in accord with Joslin's<sup>8</sup> warning, that when glycosuria is abolished and strength diminished by long fasting, and then glycosuria and acidosis allowed to return through improper diet, the last state of that man may be worse than the first. Herein may be found the

<sup>8</sup> Discussion of paper at meeting of Suffolk County Medical Society in connection with Boston Medical Library, December 2, 1914.

causes of failure in past attempts of this sort. After the fasting patient has been completely sugar-free for one or two days, feeding is begun as outlined in former papers,<sup>4</sup> and the tolerance of the patient for carbohydrate, protein, and fat is determined. Just as fasting is continued not for any limited number of days, but as long as necessary for sugar-freedom, so also the diet is governed not by any theoretical standard of protein or calories, but by the amount of each food that can be given in each individual case while keeping the urine clear. Under this program even weak and emaciated patients have been subjected to under-nutrition in both protein and calories for weeks or months continuously, with ultimate benefit. Any trace of glycosuria is the signal for a fast-day with subsequent modification of diet, and routine fast-days are often used as frequently as once a week even in absence of glycosuria. While individual details must be reserved to a later publication, two principles in the management of severe cases may be mentioned, namely, the benefit of keeping the patient permanently below weight, and the advisability of restricting the quantity of fat in the diet.

A slight temporary reduction of weight was practiced in some cases by Naunyn, whose example has been widely followed. This was not carried to the point of abolishing glycosuria and acidosis in the severest cases; and generally the attempt has been to maintain these patients at the highest possible level of weight and nutrition, with the idea of helping them to withstand a wasting disease. The present idea is the opposite to this, namely, that the reduction of weight is in itself beneficial to the diabetic condition and serves to spare the weakened function and increase tolerance. This measure, like the others, is carried to any point that may be necessary in any particular instance. Sometimes a slight reduction of weight suffices even for a severe case. Again, a well-nourished patient, easily kept free from glycosuria, was reduced by twenty kilograms merely because of a slight stubborn ketonuria and a persistently high blood-sugar. Most patients are able to regain weight to a greater or less extent, but few severe ones are able to return fully to normal weight. Any increase that is possible without return of symptoms is permit-

<sup>4</sup> Allen, F. M., Jour. Amer. Med. Assoc., September 12, 1914; Boston Med. and Surg. Jour., February 18, 1915.

ted. Any gain that brings back glycosuria or ketonuria is checked. The experience in human patients thus far runs parallel to that in the animal experiments upon which this suggestion was founded.

In the treatment of diabetes heretofore, fat has been freely given and even forced upon the patient. It has been restricted only in certain cases with high acidosis and danger of coma. The doctrine has been that fat does not affect diabetic glycosuria except in very rare "fat-sensitive" patients; also, that the diabetic must receive the number of calories required by his metabolism, plus the number of calories lost as sugar and acetone bodies in the urine, and fat has been considered the best food for crowding in these calories. Anyone can readily convince himself that, in a suitably severe diabetic who is symptom-free for days or weeks on a fixed diet, the addition of some quantity of butter or olive oil to the diet will bring back the glycosuria, ketonuria, and other symptoms immediately or within a short time. The feeding of fat alone does not cause glycosuria, and there is no proof that the sugar is formed from the fat; more probably the sugar excretion results from the stimulating effect of fat upon metabolism. The overtaxing of the patient's metabolism by giving fat beyond the limit of tolerance may be an additional explanation of the failure to keep certain patients free from glycosuria and ketonuria under former methods of treatment.

Concerning any proposed treatment, inquiry is proper whether it is new and whether its results are superior to those of the old methods. The following statements can be made regarding these points.

The principle tentatively suggested, that increase of weight or metabolism increases strain upon the internal pancreatic function and reduction of weight or metabolism reduces strain upon the internal pancreatic function, is new, and if it proves valid will be a useful general guide in treatment. The animal experiments likewise are entirely new. The general policy of insisting upon prompt and lasting freedom from glycosuria and acidosis in all cases of diabetes, even the severest, is a new one. In addition, the main features wherein the proposed treatment differs from the previously established methods may be summarized under the following five headings. The first two represent differences merely of degree, in

that the proposed treatment is more radical than the old; namely, first, an initial fast sufficient to clear up glycosuria in any case and then one or two days longer; second, a subsequent diet such as to keep glycosuria and acidosis permanently absent, with as many interspersed fast-days as necessary for this purpose. The third and fourth features represent differences not in degree but in kind, and are diametrically opposed to the prevalent teachings; that is, the third opposes the idea that the diabetic should be kept at the highest possible level of weight and strength, and that gain in weight is synonymous with improvement; and it substitutes for this the plan of keeping most severe diabetics intentionally and permanently at a sufficiently low level of weight and metabolism, in the belief that return of symptoms and downward progress is thus prevented. The fourth feature stands opposed to the doctrines that fat feeding does not appreciably influence diabetic glycosuria, and that calories lost in the urine should be replaced by additional calories in the diet, preferably in the form of fat. It opposes to these the observation that addition of fat to a fixed diet suffices to bring back both glycosuria and ketonuria in most severe diabetics, and the principle that the patient's tolerance for fat and calories should be followed in the same way as the tolerance for carbohydrate and protein. The fifth feature consists merely of routine or incidental matters, which are not without practical importance. Among these may be mentioned (1) the diet such as not to overtax tolerance and yet satisfy the patient sufficiently that he will follow it continuously at home; (2) the absence of any specific craving for carbohydrate such as diabetics are supposed to manifest, and the contradiction of the prevalent idea that most severe diabetics cannot be trusted; (3) the avoidance of the need of alkali for more than a few days, and therewith relief from disturbances due either to acidosis on the one hand or to prolonged large doses of soda on the other; (4) the principle of clearing up the urine quickly and devoting the greater part of the stay in hospital to educating the patient, rather than devoting the greater part of the stay in hospital to clearing up the urine and dismissing the patient shortly thereafter; (5) instruction of the patient in the simple means of controlling his own condition, through his



diet, his body weight, and the daily testing of his own urine with Benedict's solution.

The immediate results as observed up to the present have appeared uniformly beneficial. Reports from a number of clinicians experienced in the older methods of treating diabetes agree that these results under the new method are more favorable. Also the relative simplicity of the proposed method, and the fact that it stops glycosuria without running any risk of acidosis, makes it available for a large body of general practitioners who have heretofore not felt safe in withdrawing carbohydrate or attempting to stop glycosuria in cases with any marked ketonuria. Patients also generally accept radical treatment with quick decisive results more readily than the weeks or months of privation heretofore used in stopping glycosuria, and the quick relief from polyphagia, polydipsia, and other symptoms aids further in securing their coöperation. For these reasons it is believed that the innovations described are of some real value. As respects the remote results and the influence on the ultimate prognosis of severe diabetes, longer experience must decide. A question is here involved whether diabetes is an inherently progressive disease or whether it is the simple weakness of a metabolic function. If it is the former, patients must ultimately go down hill and die, though not in diabetic coma, and the benefit will consist only in lengthening their lives and keeping them more comfortable. If it is the latter, downward progress may be indefinitely prevented by avoiding overstrain of the weak function, just as in animals. Among the patients treated thus far, during a variable number of months in the hospital and at home, spontaneous downward progress has not yet been observed. Whatever the ultimate outcome, two conclusions seem justified by present knowledge: (1) that this treatment removes glycosuria and acidosis more quickly and surely than has been the practice heretofore, and (2) that patients do better when glycosuria and acidosis are removed than when they are allowed to continue.

## THE CHEMOSEROTHERAPY OF EXPERIMENTAL PNEUMOCOCCAL INFECTION.

By HENRY F. MOORE, M.B., B.Ch., B.A.O.

(From the Hospital of The Rockefeller Institute for Medical Research.)

(Received for publication, July 8, 1915.)

In a former communication (1) we reported that the action of ethylhydrocuprein (a derivative of hydroquinine introduced by Morgenroth and Levy (2)) was well marked *in vitro* and *in vivo* on type strains of the four groups of pneumococci. Since we possess in certain types of antipneumococcus serum the means of conferring on experimental animals considerable specific protection against very many multiples of the minimal lethal dose of a virulent pneumococcus culture, it seemed to us of interest to study the combined effect of chemo- and serotherapy *in vivo*, and to endeavor to get some numerical values representing the results.

Neufeld (3) showed that strains of pneumococci differed among themselves in respect to their immunity reactions (protective antibodies).

The work of Cole (4) and of Dochez and Gillespie (5) has resulted in a serological classification of the pneumococci. The pneumococci can be divided into at least four groups; an immune serum produced against any member of Group I has a specific agglutinative action upon, and a specific protective action against, any member of Group I, but has no such effects on any member of the other three groups. In like manner, an immune serum against any member of Group II behaves similarly with respect to any member of Group II, but has no effect on any member of Groups I, III, or IV. To Group III belong all the microorganisms of the *Pneumococcus mucosus* type. To Group IV belong all those strains of pneumococci which do not fall into the other three groups; an immune serum produced against any member of this group has a specific agglutinative action upon, and a specific protective action against, the strain used for its production, but has no such effects on any other member of this group, or of the other groups.

Neufeld and Engwer (6) and Engwer (7) have studied the effect of combining the serum and chemotherapy in guinea pigs infected with pneumococcal pleural exudates; these observers saw an increased effect by the combination. Their protocols do not show, however, to what group either the pneumococcus or antiserum belonged; it is not clear whether or not the antiserum used was

produced against a member of the group to which the infecting pneumococcus belonged. Boehncke (8), too, saw a markedly increased effect in mice by the combination of serum and drug therapy over and above the effect of either of these two separately; in the protocols of his experiments, also, we have little information as to the group to which the pneumococcus or antiserum belonged.

We deemed it of importance to study the results of combined chemotherapy and serum therapy in the light of the serological classification of the pneumococci mentioned above; and, since the results of serum therapy in man have not been at all as satisfactory in the case of lobar pneumonia due to pneumococci belonging to Group II as when the disease is caused by a strain of Group I, we have given the greater part of our attention, in this respect, to infection with pneumococci belonging to Group II. Further, the threshold value of our immune horse serum to Group I is more than ten times greater than the corresponding serum to Group II. Hence a satisfactory result with an antiserum to Group II should mean an even better result with an antiserum to Group I, in the case of infection with a strain of the homologous group.

Since toxic symptoms have been several times noticed in human patients and in experimental animals treated with ethylhydrocuprein, we have endeavored to reduce to a minimum the amount of ethylhydrocuprein given to the animals in the present study. The dose of ethylhydrocuprein (optochin base) recommended by Morgenroth (9) in experimental pneumococcal infection in the mouse is 0.5 cc. of a 2 per cent solution in olive oil given under the skin of the back immediately following the infection, and repeated on the next day; this is followed on the third day, and on the fourth day if desirable, by a similar injection of 0.4 cc. of the same solution. The dosage used in the present study is considerably below this. The experimental animals used were mice.

#### EXPERIMENTAL.

The strain of pneumococcus of Group II used in the present study was a stock strain and was maintained in a condition of maximal virulence throughout the experiments; that is to say, 0.000,001 cc. of a twenty hour broth culture regularly killed mice within forty-eight hours. Several controls of virulence were done in the case of each

experiment. A twenty hour broth culture of the pneumococcus was used in every case. In the designation of the culture, the Roman numeral represents the group to which the pneumococcus belongs, the Arabic figure the number of animal passages which the strain had had, and the exponent the number of cultivations on artificial media since the last animal passage.

Mice of 18 grams' weight and upwards were used; the dosage of the drug was calculated in every case according to the weight of the animal. An autopsy was performed on every mouse that succumbed, unless otherwise stated in the protocols (owing to the body having been eaten by the survivors).

A constant amount of immune horse serum, 0.2 cc., was used in the experiments; it was mixed with the infecting dose of culture, and the mixture, making a total volume of 1 cc., was given intraperitoneally.

The ethylhydrocuprein (optochin base) was administered in the form of a 2 per cent solution in olive oil, given under the skin of the back. This treatment was given, in the first instance, immediately

*Experiment 1. Titer of Antipneumococcus Serum II.*—(Table I.) This serum protected regularly against 0.001 cc. of a 20 hour broth culture of any strain of pneumococcus belonging to Group II.

TABLE I.  
*Culture II 42<sup>b</sup>; Virulence Maximal.*

Amount of culture.	Result.	Amount of culture.	Result.	Amount of culture.	Result.
cc.		cc.		cc.	
0.001	S	0.02	■ 48 hrs.	0.08	■ 24 hrs.
"	"	"	■ 60 "	"	■ " "
"	"	0.04	S	"	■ " "
"	"	"	"	"	■ " "
"	"	"	■ 48 "	"	■ " "
"	"	"	■ " "	"	■ " "
"	"	0.06	■ 24 "	"	■ " "
0.01	"	"	■ 48 "	"	■ 48 "
"	"	"	■ " "	"	S
"	"	"	■ " "	0.1	■ 24 "
"	"	"	■ " "	"	■ " "
"	■ 72 hrs.	"	■ " "	"	■ " "
"	■ " "	"	■ 72 "	"	■ 48 "
0.02	3	"	■ " "	"	■ " "
"	"	"	■ " "	"	■ " "
"	■ 30 "	"	■ " "		

after the infection; if a second dose was given it was given after an interval of 24 hours.

In the protocols the letter S stands for survival of the corresponding animal; a black square (■) means death of the animal from pneumococcal septicemia, capsulated diplococci having been found in its heart's blood by His's capsule stain, or, if this examination proved negative, Gram-positive diplococci having been recovered by culture (abundant inoculation on defibrinated rabbit blood agar) from the heart's blood at autopsy. An oblique cross (X) means death of the animal, the bacteriological examination of the heart's blood (and peritoneal cavity, if necessary) by smear and culture being negative; in this case the death of the animal may have been due to fortuitous influences, the toxicity of the drug, traumatism inflicted in the experiment, etc. When an autopsy was not possible on an animal, its death is indicated by the sign +.

*Comment on Experiment 1.*—From the protocol it is seen that, while the intraperitoneal injection of 0.2 cc. of antipneumococcus serum II gives to mice a sure protection against 0.001 cc. of a virulent culture (Strain II 42<sup>b</sup>), this amount of serum only gives a very doubtful protection against 0.01, 0.02, and 0.04 cc. of culture, and none at all against 0.06 cc. or more.

*Experiment 2.*—Effect of a single dose of 0.45 cc. and of a single dose of 0.5 cc., respectively, of a 2 per cent solution of ethylhydrocuprein (optochin base) in olive oil per 20 gm. of mouse on pneumococcal infection. (Table II, Strain II 42<sup>b</sup>.)

TABLE II.

Mouse No.	Amount of culture.	Normal horse serum.	Ethylhydrocuprein 2 per cent solution.	Result.	
				2d day.	3d day.
1	cc. 0.08	cc.	cc. 0.45	Sick	■
2	"	"	"	"	■
3	"	—	"	"	■
4	"	—	"	■	
5	"	0.2	"	Sick	■
6	"	"	"	"	■
7	"	"	"	"	■
8	"	"	"	"	■
9	0.001	—	0.5	"	■
10	"	—	"	"	■
11	"	—	"	Well	■
12	0.0001	—	"	Sick	■
13	"	—	"	■	
14	"	—	"	Well	■

### 372 Chemoserotherapy of Experimental Pneumococcal Infection.

*Comment on Experiment 2.*—A single treatment with either 0.45 cc. or 0.5 cc. of the 2 per cent solution of the drug is without effect on the lethal issue due to pneumococcal septicemia, not alone when the dose of infection approaches that used in the following experiments (0.08 cc. to 0.5 cc.), but also when it is 800 to 5,000 times smaller (0.0001 cc.).

*Experiment 3. Combined Therapy.*—(Table III.) Strain II 42<sup>6</sup>. Treatment: 0.45 cc. of a 2 per cent solution of ethylhydrocuprein in oil, per 20 gm. of mouse, and 0.2 cc. of antipneumococcus serum II.

TABLE III.

Mouse No.	Dose of culture.	2d day.	3d day.	4th day.	5th day.	6th day.	7 h day.	8th day.
1	cc. 0.08	Well	Well	Well	Well	Well	Well	Well.
2	"	"	"	"	"	"	"	"
3	"	"	"	"	"	"	"	"
4	"	"	"	"	"	"	"	"
5	"	"	"	"	"	"	"	"
6	"	"	"	"	"	"	"	"
7	"	"	"	"	"	"	"	"
8	"	"	"	"	"	"	"	"
9	"	"	X	"	"	"	"	"
10	"	"	Well	"	■	"	"	"
11	0.1	"	"	"	Well	"	"	"
12	"	"	"	"	"	"	"	"
13	"	"	"	"	"	"	"	"
14	"	"	"	"	"	"	"	"
15	0.2	"	"	"	"	"	"	"
16	"	"	"	"	"	"	"	"
17	"	"	"	"	"	"	"	"
18	"	"	"	■	"	"	"	"

*Experiment 3 a. Controls to Experiment 3.*—Table IV shows (1) controls infected, and treated with 0.2 cc. of antipneumococcus serum alone; (2) controls of virulence.<sup>1</sup>

TABLE IV.

(1) Controls treated with 0.2 cc. of antipneumococcus serum alone.			(2) Controls of virulence.		
Mouse No.	Dose of culture.	Result.	Mouse No.	Dose of culture.	Result.
1	cc. 0.06	■ 24 hrs.	1	cc. 0.00001	■ 48 hrs.
2	"	■ 48 "	2	0.000001	■ " "
3	0.08	■ " "	3	"	■ " "
4	"	■ 24 "	4	"	■ " "
5	"	■ " "	5	"	■ " "
6	0.1	■ " "			

<sup>1</sup> For controls treated with ethylhydrocuprein see Experiment 2.

*Comment on Experiments 3 and 3 a.*—8 of 10 animals infected with 0.08 cc. of culture and treated with 0.45 cc. of the 2 per cent solution of the drug and 0.2 cc. of serum (each of which of itself is without effect with this dose of culture) survived. One animal died on the third day and pneumococci were not recovered from the heart's blood. The remaining animal died of pneumococcal septicemia on the fifth day (delayed death). All the mice infected with 0.1 cc. of culture and similarly treated survived; 50 per cent of the series infected with 0.2 cc. of culture and also similarly treated died of pneumococcal septicemia; the others survived. The control animals all died of pneumococcal septicemia within forty-eight hours.

*Experiment 4. Combined Therapy.*—(Table V.) Strain II 43<sup>a</sup>. Treatment: 0.45 cc. of a 2 per cent solution of ethylhydrocuprein in oil per 20 gm. of mouse, and 0.2 cc. of antipneumococcus serum II.

TABLE V.

Mouse No.	Dose of culture.	2d day.	3d day.	4th day.	5th day.	6th day.
	cc.					
1	0.3	Well	Well	■		
2	"	"	"	■		
3	"	"	"	■		
4	"	"	"	■		
5	"	"	"	■		
6	"	Sick	+ (eaten)			
7	0.5	Well	Well	Well	Well	Well.
8	"	"	"	"	"	"
9	"	"	+ (eaten)			
10	"	■				
11	0.7	Well	Well	"	"	"
12	"	"	■			
13	0.8	"	Well	Sick	■	
14	"	"	"	■		

*Comment on Experiment 4.*—In this experiment in which a larger dose of infection was given than in Experiment 3 the results did not come out uniformly or clear. The majority of the animals died, although in most cases death was delayed. We thought that a small dose of optochin given on the second day might serve to tide the animals over the third and fourth days and so help ultimate re-

### 374 *Chemotherapy of Experimental Pneumococcal Infection.*

covery; the result of this modification in dosage of the drug is shown in the next experiment. Virulence controls were done, as in Experiment 3 a, but are not shown in the table.

*Experiment 5. Combined Therapy.*—(Table VI.) Strain II 43<sup>b</sup>. Treatment: 0.45 cc. of 2 per cent ethylhydrocuprein in oil immediately after infection and 0.4 cc. after 24 hours' interval per 20 gm. of mouse; 0.2 cc. of antipneumococcus serum II at the time of infection.

TABLE VI.

Mice No.	Dose of culture.	2d day.	3d day.	4th day.	5th day.	6th day.
1	cc.	Well	Well	Well	Well	Well.
2	0.3	"	"	"	"	"
3	"	"	"	"	"	"
4	"	"	"	"	"	"
5	"	"	"	"	"	"
6	"	"	"	"	■	
7	"	"	"	"	X	
8	"	"	"	■		
9	"	Sick	+ (eaten)			
10	0.5	Well	Well	Well	Well	"
11	"	"	"	"	"	"
12	"	"	"	"	■	
13	"	"	"	"	■	
14	"	"	"	■		
15	"	"	"	■		
16	"	"	Sick	■		
17	"	Sick	+ (eaten)			

*Comment on Experiment 5.*—50 per cent of the animals infected with 0.3 cc. of culture, and treated as stated, survived; of those that died, pneumococci were not recovered from the heart's blood of one; one was eaten by its fellows and could not, therefore, be autopsied; two gave positive blood cultures. In the case of infection with 0.5 cc. of culture, only two animals recovered; pneumococci were recovered from the heart's blood of all those that died except one, the body of which was so far eaten that it could not be bacteriologically examined. In this experiment the altered dosage of the drug ensured a better result than in the former case, but it is evident that we are at the limit of protective power for the conditions obtaining therein. Consequently we proceeded to study the effect of a single larger dose of drug combined with the serum therapy; namely, 0.5 cc. of the 2 per cent oily solution per 20 grams of mouse. Virulence controls were done, as in Experiment 3 a, but are not shown in the table.



*Experiment 6. Combined Therapy.*—(Table VII.) Strain II 44<sup>4</sup>. Treatment: 0.5 cc. of 2 per cent ethylhydrocuprein base in oil per 20 gm. of mouse, and 0.2 cc. of antipneumococcus serum II.

TABLE VII.

Mouse No.	Dose of culture.	2d day.	3d day.	4th day.	5th day.	6th day.	7th day.	8th day.
	cc.							
1	0.2	Well	Well	Well	Well	Well	Well	Well.
2	"	"	"	"	"	"	"	"
3	"	"	"	"	"	"	"	"
4	"	"	"	"	"	"	"	"
5	"	"	"	"	"	"	"	"
6	"	"	"	"	"	"	"	"
7	"	"	"	"	+ (eaten)			
8	"	+ (eaten)						
9	0.3	Well	"	"	Well	"	"	"
10	"	"	"	"	"	"	"	"
11	"	"	"	"	"	"	"	"
12	"	"	"	"	"	"	"	"
13	"	"	"	"	"	"	"	"
14	"	"	"	"	"	"	"	"
15	"	"	"	+ (eaten)				
16	"	"	"	"				
17	0.4	"	"	Well	"	"	"	"
18	"	"	"	"	"	"	"	"
19	"	"	"	"	"	"	"	"
20	"	"	"	"	"	"	"	"
21	"	"	"	"	"	"	"	"
22	"	"	"	"	"	"	"	"
23	"	"	"	"	"	"	+ (eaten)	
24	"	"	"	X				
25	"	■						

Two mice infected with 0.000001 cc. and one with 0.00001 cc. of Strain II 44<sup>4</sup> (controls of virulence) died of pneumococcal septicemia in thirty-six hours. Thirteen controls infected with 0.08 cc. of culture and treated with 0.5 cc. of the ethylhydrocuprein solution alone immediately after the infection all died on the third day (forty-eight hours after infection) from pneumococcal septicemia.

*Comment on Experiment 6.*—In this case we increased the single dose of ethylhydrocuprein to 0.5 cc. of the 2 per cent solution of the base in oil per 20 grams of mouse. Six of eight animals infected with 0.2 cc. of culture, and six of eight animals infected with 0.3 cc. of culture, and treated as stated, survived. The four mice which died were eaten by their fellows and therefore could not be autopsied; in three of these cases death was delayed (fourth and fifth

days). Of the nine animals infected with 0.4 cc. of culture, six survived; one died on the second day with a sterile heart's blood (abundant inoculation) but pneumococci were recovered from the peritoneal cavity; one died on the fourth day and showed a sterile heart's blood on cultivation; and one was eaten by its fellows on the seventh day. The thirteen controls infected with 0.08 cc. of culture and treated with the dose of drug used in the actual experiment all died in forty-eight hours, pneumococci being present in the heart's blood of all. These controls are not shown in the protocol.

*Experiment 7. Combined Therapy.*—(Table VIII.) Strain II 45<sup>3</sup>. Treatment: 0.5 cc. of 2 per cent solution of ethylhydrocuprein base in oil per 20 gm. of mouse, and 0.2 cc. of antipneumococcus serum II.

TABLE VIII.

Mouse No.	Dose of culture.	2d day.	3d day.	4th day.	5th day.	6th day.	7th day.	8th day.
1	0.4	Well	Well	Well	Well	Well	Well	Well.
2	"	"	"	"	"	"	"	"
3	"	"	"	"	"	"	"	"
4	"	"	"	"	"	"	"	"
5	0.5	"	"	"	"	"	"	"
6	"	"	"	"	"	"	"	"
7	"	"	"	"	"	"	"	"
8	"	"	"	"	"	"	"	"
9	"	"	"	"	"	"	"	"
10	"	Sick	"	"	"	"	"	"
11	"	"	"	"	"	"	"	"
12	"	"	"	■	"	"	"	"
13	0.6	"	"	Well	"	"	"	"
14	"	"	"	"	"	"	"	"
15	"	"	"	"	"	"	"	"
16	"	Sick	Sick	Sick	■	"	"	"
17	"	Well	■	"	"	"	"	"
18	"	Sick	■	"	"	"	"	"

Three virulence controls (0.000001 cc.) all died in forty-eight hours of pneumococcal septicemia.

*Comment on Experiment 7.*—All the animals infected with 0.4 cc. of culture and treated, and all but one infected with 0.5 cc. of culture and treated, survived; from the heart's blood of the one which died (fourth day) pneumococci were recovered. 50 per cent of those infected with 0.6 cc. of culture and treated survived; the

remainder died of pneumococcal septicemia (third to fifth day). We have here, evidently, reached the limit of protection to be gotten by the simultaneous administration of antiserum and drug in the manner and quantities indicated above.

In addition to the experiment mentioned above, we have carried out a series of experiments in which the mice were treated with ethylhydrocuprein and a non-homologous serum. For example, we have used an antiserum to pneumococcus of Type I in the case of infection with from 0.0001 cc. to 0.1 cc. of a broth culture of a virulent pneumococcus of Type II (Strain II 34<sup>20</sup>), the minimal lethal dose of which was 0.000001 cc.; and we have given the ethylhydrocuprein solution both as one dose (0.5 cc.) at the time of infection, and as three doses on the three first days of the experiment (0.25 cc., 0.25 cc., and 0.2 cc.), the administration of the first dose following the infection immediately; by thus combining the drug treatment with non-homologous serum therapy, we have seen no effect such as we have described above when the serum was homologous. Again, in the case of treatment of infection with a virulent strain of Group IV (Strain IV A 67.11<sup>8</sup>), the minimal lethal dose of which was 0.000001 cc., we have had no such success on giving the drug and a potent antiserum to pneumococci of either Group I or Group II. The protocols of these experiments need not be exhibited.

#### DISCUSSION OF RESULTS.

The results of this study of the treatment of experimental pneumococcal infection in the mouse by simultaneous administration of ethylhydrocuprein and antipneumococcus serum are clear. The protocols show that 0.5 cc. of a 2 per cent oily solution of ethylhydrocuprein (optochin base), or 0.2 cc. of the type homologous antiserum, are each, by themselves, powerless to protect against 0.06 cc. of a highly virulent culture of a pneumococcus of Group II; but that if both these bodies, which are chemically far removed from each other, be exhibited simultaneously, protection is given to the animal against as large a dose of infection as 0.5 cc. of culture. The potency of the antiserum of Type II is such that 0.2 cc. confers a certain protection on mice against simultaneous infection with an amount of a virulent culture lying somewhere between 0.001 cc. to

### 378 *Chemotherapy of Experimental Pneumococcal Infection.*

0.01 cc.; with a higher dosage of culture this protection is quite uncertain, while it is altogether absent in the case of infection with 0.06 cc. of culture. The dose of ethylhydrocuprein generally used in the present study (0.5 cc. of the 2 per cent solution) is unable to protect against a dose of infection as small as 0.0001 cc. of a virulent culture. Hence, the exhibition of such a small dose of this drug as 0.5 cc. of the 2 per cent solution (0.01 of a gram) per 20 grams of mouse is capable of raising the threshold value of the serum more than fifty times; that is to say, mice are protected against 0.5 cc. of a virulent culture by the combined therapy, while 0.2 cc. of serum alone will not invariably protect against 0.01 cc. of culture, and the dose of ethylhydrocuprein used does not even protect against five thousand times less than 0.5 cc. of culture. Thus the protective value of simultaneous administration of the drug and serum, even though they be given by different routes, is many times greater than,

TABLE IX.

*Combined Ethylhydrocuprein and Serum Therapy.*

*Type II Antiserum. Pneumococcus, Stock Strain of Group II.*

Culture.	Ethylhydrocuprein 2 per cent solution.	Antiserum II.	Normal horse serum.	Survivals.	Deaths due to pneumococcal septicemia.	Deaths due to doubtful cause (no autopsy).
cc.	cc.	cc.	cc.	per cent	per cent	per cent
0.001	—	0.2	—	100	0	0
0.01	—	"	—	66.6	33.3	0
0.02	—	"	—	40	60	0
0.04	—	"	—	60	40	0
0.06	—	"	—	0	100	0
0.08	—	"	—	0	100	0
0.08	0.45	—	—	0	100	0
"	"	—	0.2	0	100	0
"	0.5	—	—	0	100	0
"	"	—	0.2	0	100	0
0.0001	"	—	—	0	100	0
0.08	0.45	0.2	—	80	10	10
0.1	"	"	—	100	0	0
0.2	"	"	—	50	50	0
0.3	"	"	—	0	100	0
0.2	0.5	0.2	—	75	0	25
0.3	"	"	—	75	0	25
0.4	"	"	—	76.9	7.6	15.3
0.5	"	"	—	87.5	12.5	0
0.6	"	"	—	50	50	0

and out of all proportion to, the protective value of either alone. The preceding epitome of the results (Table IX) will make this clear.

It is important to note that, where a non-homologous antiserum is used in this combined method, *e. g.*, a Type I antiserum with a pneumococcus of Group II or a Type I or II antiserum with a pneumococcus of Group IV, no such effect as that just stated is seen. Indeed, no more effect is obtained than if normal, and not immune, serum be used. It seems that, if the dose of ethylhydrocuprein given in the first instance be below a certain quantity in relation to the amount of the infection, subsequent readministration of the drug on the next day is powerless to prevent a fatal result from pneumococcal septicemia.

#### CONCLUSIONS.

1. A single small dose of ethylhydrocuprein (optochin base), which by itself has practically no protective effect against experimental pneumococcal infection in mice, is capable of increasing the threshold value of the type homologous antipneumococcus serum at least fifty times.

2. This effect is proportionately many times greater than a simple summation of the protective effects of these two bodies.

3. No such effect is obtained when the antiserum used is one produced against a strain of pneumococcus from a group other than that to which the infecting pneumococcus belongs.

#### BIBLIOGRAPHY.

1. Moore, H. F., *Jour. Exper. Med.*, 1915, xxii, 269.
2. Morgenroth, J., and Levy, R., *Berl. klin. Wchnschr.*, 1911, xlviii, 1560, 1979.
3. Neufeld, F., and Haendel, *Ztschr. f. Immunitätsforsch., Orig.*, 1909, iii, 159; *Berl. klin. Wchnschr.*, 1912, xlix, 680; *Arb. a. d. k. Gsundtsamte*, 1910, xxxiv, 169; in Kolle, W., and von Wassermann, A., *Handbuch der pathogenen Microorganismen*, 2d edition, Jena, 1913, iv, 513.
4. Cole, R., *Arch. Int. Med.*, 1914, xiv, 56.
5. Dochez, A. R., and Gillespie, L. J., *Jour. Am. Med. Assn.*, 1913, lxi, 727.
6. Neufeld and Engwer, *Berl. klin. Wchnschr.*, 1912, xlix, 2381.
7. Engwer, T., *Ztschr. f. Hyg. u. Infektionskrankh.*, 1913, lxxiii, 194.
8. Boehncke, K. E., *München. med. Wchnschr.*, 1913, lx, 398.
9. Morgenroth, J., and Kaufman, M., *Ztschr. f. Immunitätsforsch., Orig.*, 1913, xviii, 145.

## A CHEMICAL STUDY OF WOMAN'S MILK, ESPECIALLY ITS INORGANIC CONSTITUENTS.\*

By L. EMMETT HOLT, M.D., ANGELIA M. COURTNEY, AND  
HELEN L. FALES.

*(From the Laboratories of The Rockefeller Institute for Medical Research  
and the Babies' Hospital.)*

No extended review of the literature on the chemistry of woman's milk will be attempted in this paper. The reader is referred for this to a résumé by Nothmann<sup>1</sup> in 1912 and another by Talbot<sup>2</sup> in 1914. Quite large series of analyses have been made by many observers in which only a determination of the organic constituents and the total ash of milk was aimed at. Among the most important publications are those of Leeds,<sup>3</sup> Meigs<sup>4</sup> and Adriance<sup>5</sup> in this country and those of Pfeiffer<sup>6</sup> and Schlossmann<sup>7</sup> in Germany.

The fat and protein content of woman's milk are now pretty definitely agreed on. The methods followed by many chemists in the sugar determination, as we shall see later, are open to criticism; and even in the total ash estimation, errors have been made owing to faulty methods.

Considering how much work has been done on the chemistry of woman's milk, it is surprising that so few investigators have concerned themselves with the composition of the ash. With the exception of Harrington and Kinnicutt<sup>8</sup> who analyzed a single large com-

\* Read at the meeting of the American Pediatric Society, Lakewood, N. J., May 26, 1915.

1. Nothmann: *Jahrb. f. Kinderh.*, 1912, lxxv, 724.
2. Talbot: *AM. JOUR. DIS. CHILD.*, 1914, vii, 445.
3. Leeds: Reprint, *Proc. Am. Assn. Adv. Sc.*, 1884.
4. Meigs: *Milk Analysis and Infant Feeding*, Phila., 1885.
5. Adriance: *Arch. Pediat.*, 1897, xiv, 22, 85.
6. Pfeiffer: *Verhandl. d. Versamml. d. Ges. f. Kinderh.*, Wien, 1894, p. 126.
7. Schlossmann: *Arch. f. Kinderh.*, 1900, xxx, 288.
8. Harrington and Kinnicutt: *Rotch, Pediatrics*, 1906.

posite sample, almost nothing has been published in this country and very little abroad on this subject. It is true that both here and abroad isolated analyses have been reported, but they have been mainly in connection with metabolism experiments and have frequently been made when the conditions were presumably pathologic. Even the writers who have undertaken to study the normal composition of the ash in woman's milk and who have been most extensively quoted in literature have made a surprisingly small number of observations.

The earliest modern investigator was Bunge<sup>9</sup> who published in 1874. Though his methods were exact, practically those in use today, so that his results can be relied on, yet his figures which have been so widely quoted were derived from two examinations of the milk of the same woman, one made on the fifteenth and the other on the eighteenth day of lactation. He used large samples, but he does not state that it was the 'twenty-four hours' secretion. Söldner,<sup>10</sup> whose work is also much quoted, published but six analyses, three of these being of colostrum and none being of milk at a later period than three and a half months. In only one instance was his examination of an individual sample. Pelka's<sup>11</sup> observations were but two in number, both made on composite samples. The single observations by Blaubeurg,<sup>12</sup> Abderhalden,<sup>13</sup> Birk<sup>14</sup> and de Lange<sup>15</sup> practically complete the list to which reference is made in literature on the salts of woman's milk up to the work of Schloss<sup>16</sup> in 1912.

The most important recent contribution on this subject has been made by the author last mentioned. His observations are much more numerous than those of any of the other writers. They were made on ten large individual samples of milk, eight of these being the total secretion for twenty-four hours; the other two were large samples collected during several successive days. In addition, two

9. Bunge: *Ztschr. f. Biol.*, 1874, x, 295.

10. Camerer and Söldner: *Ztschr. f. Biol.*, xxxix, 173; xlv, 61.

11. Pelka: *Ztschr. f. Kinderh.*, 1911, ii, 442.

12. Blaubeurg: Quoted by Engel in *Sommerfeld's Handbuch der Milchkunde*, Wiesbaden, 1909, 800.

13. Abderhalden: Personal communication quoted by Schloss.

14. Birk: *Monatschr. f. Kinderh.*, 1910-11, ix, 595.

15. De Lange: *Ztschr. f. Biol.*, 1900, xl, 527.

16. Schloss: *Monatschr. f. Kinderh.*, 1910-11, ix, 636; x, 499.

composite samples, obtained respectively from fifteen and sixteen women, were examined.

Schloss divides lactation into three periods: (1) colostrum period; (2) transition period extending from the colostrum to the end of the fourth week; (3) mature, or as he terms it, the period of "ripe" total ash and ash constituents, show great differences in individuals, the ratio of the separate constituents to one another is fairly constant. He was particularly struck by the parallelism of the total nitrogen and total ash values. From his findings he concludes that, with the exception of fat, all the constituents of milk stand in a fairly definite ratio to one another. The high protein and high ash of colostrum milk, established by the earlier investigators, were confirmed by his findings. After the colostrum period these values sink rapidly, but no falling off was shown in his four mature cases taken between the twenty-sixth day and four and a half months. It was only in the milk of three women examined after ten months that a marked reduction was evident. There was no evidence to prove a relationship between the daily quantity and concentration.

In only one case were there high figures with a relatively small production of milk. The great variation in previously published figures, especially of calcium, he explains by the fact that most authors have analyzed only small portions of the twenty-four hours' secretion, and in many cases the milk was from mothers of rachitic children. Schloss concludes that there are not yet enough analyses, even including those of all previous workers, to establish a definite picture of the composition of woman's milk, particularly of the ash in the different periods of lactation.

Besides the work referred to on the entire ash, a number of authors have investigated separate salts of milk. Thus, a special study of the phosphorus has been made by Schlossmann.<sup>17</sup> His results from the analyses of the milk of thirty-one individual women show the following averages:

Colostrum period, 2 women .....	.0533
Transition period, 6 women .....	.0463
Mature period, 21 women .....	.0460
Late period, 2 women .....	.0360

17. Schlossmann: Arch. f. Kinderh., 1905, x1, 1.



The calcium content of milk in normal and various abnormal conditions has been studied quite exhaustively by Bahrdt and Edelstein.<sup>18</sup> They give results obtained by eight other investigators of normal milk, but the figures are chiefly from their own observations. They give the following values for CaO in the milk of eighty individual mothers of healthy children taken at the different periods of lactation, exclusive of authors whose results we have tabulated in our own paper. They may be grouped as follows:

Colostrum period, 4 women .....	.0476
Transition period, 23 women .....	.0397
Mature period, 51 women .....	{ 1- 3 months ( 7) .0489
	{ 3- 5 months (32) .0411
	{ 6- 8 months ( 6) .0385
	{ 8-10 months ( 6) .0392
Late period, 2 women .....	{ 14 months (1) .0452
	{ 16 months (1) .0341

In these figures it will be noted there is very little variation after the colostrum period.

The iron content of woman's milk has also been studied by Bahrdt and Edelstein.<sup>19</sup> They employed the method of Neumann, in which the iron is brought down by a zinc reagent, filtered off, and dissolved in HCl; potassium iodid is then added and the freed iodine titrated with sodium thiosulphate. The amounts used for single determinations varied from about 708 c.c. to 1,300 c.c. The samples were made up of portions collected through periods of from two to four days. The milk of three nurses was studied at different times; the periods of lactation in all were between the twenty-fourth day and the sixth month. They obtained a range of .00012 to .00029 per cent. with an average value for Fe<sub>2</sub>O<sub>3</sub> of .00017 per cent. The individual variations were slight.<sup>20</sup>

These figures for iron correspond closely with those of Söldner

18. Bahrdt and Edelstein: *Jahrb. f. Kinderh.*, 1910, lxxii, Supplement, p. 16.

19. Bahrdt and Edelstein: *Ztschr. f. Kinderh.*, 1910-11, i, 182.

20. Edelstein and Csonka (*Biochem. Ztschr.*, 1912, xxxviii, 14) have recently studied the iron content of cow's milk, using large samples for analysis. When milk was carefully drawn they obtained a range of values from 0.4 to 0.7 mg. per liter, with an average of 0.5. They conclude that the iron in cow's milk is only about one-third that in woman's milk. In point of fact the amount is so small as to be negligible.

who from examinations of two large samples of milk taken between the third and twelfth days gives values of .00021 and .00013—an average of .00017 per cent. Iron values given by several writers are considerably higher than those quoted; but many of them have been obtained from such small samples that the results of the analyses cannot be relied on.

From the great interest which has recently developed in the metabolism of the salts, it seemed to us desirable that the question of the composition of the ash in woman's milk should be studied anew and on a more extended scale than hitherto, and that further, an effort should be made to learn more of the individual variations in the different salts by the use of large individual samples.

The chief purpose of the investigation, the results of which are herewith presented, was a study of the composition of the ash in woman's milk at the different periods of lactation. Incidentally, we have made determinations of the other constituents of milk—fat, sugar and protein—and have made a study of the methods commonly employed in milk analysis.

In all, examinations have been made of thirty-two large individual samples—in most cases the entire twenty-four hours' secretion—and of six composite samples. Following the classification suggested by Schloss, these cases have been grouped as follows: In the "colostrum" period, two individual and three composite samples; in the "transition" period, three individual and three composite samples; in the "mature" period (one to nine months) seventeen individual samples. We have classed separately in a "late" period ten cases of milk from the tenth to the twentieth month. As we were endeavoring to secure normal values, only the milk of apparently healthy women with healthy children was taken.

The value of the observations it seems to us is enhanced by the fact of the large individual samples obtained for analysis. Of those who have previously studied this subject only Schloss appears to have appreciated the importance of securing for examination the twenty-four hours' secretion from a single individual. The observations we have made on the milk of the colostrum and transition periods have not quite the same importance as those of mature milk, since in a number of instances only composite samples were available for analysis.

The results of many of the published analyses of breast milk are open to criticism, since such small samples were used for study. This applies of course chiefly to the ash, but it has also some importance in the estimation of the other constituents. As already suggested, the methods employed by many authors are now known to be unreliable, while others make no mention of methods used. For comparison with our own results we have brought together the figures for ash analyses from the other investigators in this field whose methods could be relied on.

### *Methods Employed.*

*Nitrogen.*—The *Total N* was determined in both liquid and dried samples by the Kjeldahl-Gunning method. The casein was precipitated by dilute acetic acid in a cold solution and the N determined in the filtrate, thus giving, first, the percentage of total protein which is casein, and secondly, the percentage of protein not precipitated by acetic acid. The latter figure necessarily includes any nonprotein nitrogenous substances which are present in the milk; there is evidence that such substances exist. In calculating the total protein as six and a quarter times the N there is therefore a slight error.

*Fat.*—The fat was determined in dried material by extraction with ether according to a modification of the Soxhlet method.

*Sugar.*—The question of the milk sugar content of the human milk does not seem yet to be definitely settled. Authors differ considerably in the range of values which they report, some, especially among the earlier investigators, giving figures below 6 per cent., while others obtain averages of above 7 per cent., and occasional figures run above 8 per cent.

Three general methods of sugar determinations have been employed: (1) Estimation by difference; (2) by the polariscope; (3) by some reduction method. In the first it has been customary to determine separately the fat, protein and ash and to regard the difference between the sum of these and the total dried weight as sugar. All the error in the analysis is thus thrown on the sugar. This method has been and still is much used, but is open to very obvious objections. Polariscope readings require considerable correction and are regarded as unreliable by several authors, including Reiss and Sommerfeld.<sup>21</sup> They may be too low because of the presence in the milk of substances which may cause rotation to the left.

Most of the figures for milk sugar reported have been obtained by some reduction method. Reduction methods may give too high figures because of the presence of other reducing substances than lactose. Schlossmann used a reduction method but obtained the value for the reduced copper by weighing instead of by titration. His range is from 5.2 to 10.9 per cent. Söldner used the same method and reports values from 5.35 to 7.52 per cent. If there is any difficulty due to the presence of reducing substances other than lactose, it is evidently

21. Reiss and Sommerfeld in Sommerfeld's *Handbuch der Milchkunde*, 1909.

not avoided by these investigators. Lust<sup>22</sup> gives for twenty-five women a range of 5.7 to 8.5 per cent., averaging 7.1 per cent. He also used a reduction method but made a colorimetric determination of the reduced copper. Schloss employed the titration method, but considers it inexact and does not include his results in his tables; his figures for sugar ranged from 6.38 to 7.9 per cent.

In our analyses the sugar was estimated after removal of the protein by boiling and then adding dilute acetic acid. The sugar was then determined by a titration method with Fehling's solution, in which the copper oxid is held in solution by potassium ferrocyanid. It was found that this method gave almost invariably higher results than the polariscope, but that it agreed very well with other reduction methods, including the Volhard. Both the reduction methods and the polariscope determinations are therefore also open to criticism. There cannot be said yet to have been devised a wholly satisfactory method for sugar determination in woman's milk.<sup>23</sup>

On the whole the indirect method of estimating the sugar by difference, although open to the most obvious objections, appeared in our work to give the most consistent results. If this method is followed, however, a definite procedure in drying must be employed. We have dried the samples to minimum weight over the water bath and then allowed them to come to constant weight in the air at room temperature. This last step was to make sure that the lactose when weighed should contain all its water of crystallization. Cammerer and Söldner showed that the dried matter at equilibrium with the air includes the water of crystallization of milk sugar, but that if milk is brought to a constant weight in vacuo at 98 C. the lactose is in an anhydrous condition. The figures given in our tables represent the lactose with its water of crystallization. The lactose in an anhydrous form weighs 5 per cent. less. Most authors do not state which form of lactose their figures indicate. In our table we have given both the figures obtained by the reduction method and also those in which the estimation is by difference.

*Ash.*—The greater part of the twenty-four hours' secretion was dried on a steam bath until it reached a constant weight in equilibrium with room temperature and humidity. In this dried material the total ash and the separate ash constituents were determined. The method of ashing is of considerable importance. The one employed is that described by Karl Stölte;<sup>24</sup> a sufficient sample

22. Lust: *Monatschr. f. Kinderh.*, 1912, vi, 236.

23. Since the greater part of this work was completed, a new method for removing the protein from milk, preparatory to sugar determination, has been suggested by Hill of Ithaca (*Jour. Biol. Chem.*, March, 1915). To precipitate the protein after boiling, colloidal iron is added to the specimen of milk. This method was proposed for cow's milk. In applying this to woman's milk we have found it advantageous to add a few drops of a saturated solution of magnesium sulphate. In certain samples of milk it is impossible to obtain a clear filtrate with the acetic acid precipitation. In such cases the new procedure is of much value. By means of it we have never failed to obtain a clear filtrate. The application of this method, however, would affect the result in only a very small number of the analyses given in this paper.

24. Stölte: *Biochem. Ztschr.*, 1911, xxxv, 104.

of the dried substance, finely ground, is weighed into a platinum dish, which is set on pieces of broken pipe-clay inside a porcelain dish of a diameter 1 to 2 inches greater than that of the platinum dish. Low heat from a large-sized Teklu burner is applied to the porcelain dish until the material is well charred; then the heat is gradually increased to the greatest possible point. When most of the black has disappeared, the platinum dish is covered by a piece of platinum foil and ignition continued until ashing is completed. This method seems to be especially advantageous in connection with the determination of sodium and potassium, since it is found that pure sodium chlorid and potassium chlorid subjected to this treatment for hours lose no weight whatever. Ash obtained in this way was used for the determination of calcium, magnesium, phosphorus, sodium and potassium.

To avoid the possibility of a slight loss of chlorin during the breaking down of the organic compounds, this constituent of the milk was determined directly in the dried material. We have followed the method of removal of the protein by ferric alum and nitric acid, and titration of the chlorids in the filtrate according to Volhard. This is similar to the method chosen by Schloss after he had carefully tested it in comparison with other methods commonly used.

*Calcium, magnesium, sodium, potassium and phosphorus* were all determined by the usual methods. Calcium was precipitated as oxalate and weighed as oxid. Magnesium was precipitated as magnesium ammonium phosphate and weighed as pyrophosphate. Sodium and potassium were separated as chlorids and the potassium determined in the combined chlorids by precipitation with platinic chlorid. By the use of the Stölte method of ashing just described, the danger of volatilization of potassium and sodium chlorids, both in the initial ashing and at the stage of driving off ammonium salts, is entirely avoided. Unless this or some similar precaution is taken the values obtained for potassium and sodium are absolutely unreliable. Phosphoric acid was precipitated as ammonium phosphomolybdate; this was dissolved by ammonia and magnesium ammonium phosphate, precipitated by magnesia mixture and weighed as pyrophosphate.

The fat, sugar, protein and total ash in milk of the different periods are given in Table I.

*Colostrum.*—The chief characteristic of the milk of the colostrum period is the high protein and the high total ash. The ash in one sample (No. 3) was so much lower than all the others that it must be regarded as an exceptional individual variation. Excluding this one the average ash for the period was 0.3077, the average protein 2.25 per cent. These figures correspond fairly well with those published by other observers. The specific gravity differs little from that of the milk of other periods.

Our values for sugar are somewhat higher than those reported by other investigators. The sugar figure was obtained by reduction in

TABLE 1.  
Percentage Composition of Woman's Milk.  
A. Colostrum Period (1 to 12 Days).

No.	Age of Woman, Yrs.	Age of Child, Days	No. of Child	Sample	Amt. in c.c.	Sp. Gr.	Total Solids	Fat	Sugar by		Protein			Ash
									Reduction	Difference	Total	Casein	Albumin	
1	19-21	3-4	...	Composite, 5 women	295	1.032	12.87	2.85	7.66	2.06	...	...	...	.2960
2	18-25	3-5	...	Composite, 6 women	960	1.032	12.68	3.30	6.50	7.09	1.96	.26	1.70	.3312
3	.....	5-7	...	Individual	320	1.035	12.83	2.13	7.92	2.60	...	...	...	[.1747]
4	.....	5-8	...	Individual	240	1.032	15.05	[4.43]	7.89	2.44	...	...	...	.2921
5	20-25	5-12	...	Composite, 4 women	210	.....	13.67	3.05	8.12	2.19	...	...	...	.3117

B. Transition Period (12 to 30 Days).

6	16-21	12-30	...	Composite, 4 women	285	.....	15.66	5.64	8.18	1.61	...	...	...	.2335
7	32	14	...	Individual	215	1.032	10.36	[1.33]	7.76	1.06	...	...	...	.2132
8	20	15-18	...	Composite, 2 women	490	1.032	12.74	3.89	7.24	1.36	...	...	...	.2471
9	19-32	14-28	...	Composite, 5 women	175	.....	14.18	4.01	7.88	1.99	...	...	...	.3042
10	18	21	...	Individual	175	.....	13.77	.....	1.42	...	...	...	...	.2262
11	19	21	...	Individual, 24 hours	975	1.032	13.66	3.96	7.80	7.73	1.75	.45	1.30	.2204

C. Mature Period (1 to 9 Months).

		Mos.												
12	18	1	1	Individual, 48 hours	1,075	1.024	9.81	3.07	5.54	5.44	1.12	.35	.77	.1825
13	32	2	2	Individual, 24 hours	975	1.032	10.30	1.86	8.20	7.19	1.03	.40	.63	.2200
14	24	2	3	Individual, 24 hours	1,100	1.031	11.82	2.83	7.86	7.73	1.07	.41	.66	.1902
15	26	3	2	Individual, 36 hours	1,025	1.033	10.54	1.67	8.38	7.82	0.88	.20	.68	.1717
16	25	3	2	Individual, 24 hours	800	1.036	12.16	2.93	7.05	8.06	0.96	.46	.50	.2127
17	23	3	1	Individual, 24 hours	900	.....	12.05	2.87	7.78	7.73	1.23	.51	.72	.2135
18	32	3	2	Individual, 24 hours	850	.....	14.43	4.53	7.33	8.18	1.50	.64	.86	.2254
19	27	3½	2	Individual, 24 hours	950	1.031	13.11	3.89	8.38	7.72	1.26	.46	.80	.2433
20	23	3¾	1	Individual, 24 hours	880	.....	13.45	3.74	7.27	8.36	1.16	.52	.64	.1914
21	39	4	2	Individual, 24 hours	1,050	1.033	11.31	2.59	8.45	7.50	1.01	.34	.67	.2090
22	23	4	1	Individual, 24 hours	975	1.032	12.18	3.19	8.11	7.65	1.13	.36	.77	.2141

TABLE 1.—Continued.  
C. Mature Period (1 to 9 Months).

No.	Age of Woman, Yrs.	Age of Child, Mos.	No. of Child	Sample	Amt. in c.c.	Sp. Gr.	Total Solids	Fat	Sugar by		Protein			Ash
									Reduction	Difference	Total	Casein	Albumin	
23	26	5	1	Individual, 24 hours	975	1.031	12.38	3.76	8.38	7.29	1.10	.45	.65	.2281
24	37	6	2	Individual, 48 hours	975	1.033	11.53	2.15	8.10	8.01	1.18	.36	.82	.1922
25	30	6	4	Individual, 36 hours	1,150	1.030	12.59	3.26	7.98	8.05	1.12	.53	.59	.1590
26	30	7	2	Individual, 24 hours	840	.....	14.13	5.48	7.48	7.11	1.29	.52	.77	.2402
27	22	8½	1	Individual, 48 hours	960	1.033	13.20	4.42	7.75	7.26	1.31	.53	.78	.2158
28	28	9	1	Individual, 2 days	960	1.033	11.86	3.27	7.60	7.16	1.23	.42	.81	.1967

D. Late Period (10 to 20 Months).

29	31	10	4	Individual, 4 days	450	.....	12.25	3.02	7.92	8.02	1.00	.30	.70	.....
30	25	10½	1	Individual, 2 days	465	1.031	12.29	3.24	7.58	8.00	0.84	.....	.....	.2148
31	23	11¼	1	Individual, 2 days	480	1.036	10.00	0.97	.....	7.77	1.05	.....	.....	.2107
32	33	12	3	Individual, 3 days	350	1.031	12.16	2.43	7.65	8.36	1.15	.35	.80	.2141
33	30	12½	4	Individual, ½ day	650	1.032	12.38	3.71	7.57	7.58	0.92	.40	.52	.1728
34	26	12½	..	Individual, 1 day	690	1.028	12.77	3.87	7.01	7.66	1.03	.42	.61	.2108
35	..	14	..	Individual, 3 days	560	.....	10.63	2.00	7.14	7.31	1.14	.05	1.09	.1790
36	30	15	4	Individual, ½ day	640	1.029	15.56	6.20	7.10	8.00	1.20	.64	.56	.1675
37	..	18	..	Individual, 5 days	615	1.030	12.30	3.51	7.47	7.40	1.17	.....	.....	.2251
38	35	20	6	Individual, 3 days	690	1.033	11.44	2.70	7.77	7.34	1.22	.16	1.06	.1855

but a single sample; in the others it was estimated by difference. The reason for this was that our chief purpose was a study of the salts of milk, and it was sometimes impossible to do both determinations with the amount of milk which could be obtained for examination. The fat for all the samples analyzed averaged 3.15 per cent. But one of the individual samples which was taken at the very end of the colostrum period was unusually high, 4.43 per cent. An average of the remaining four analyses, including three composite and one indi-

vidual sample, was 2.83 per cent., which is probably much nearer the usual fat content of colostrum milk. It corresponds with the results obtained by others.

*Transition.*—In the transition period there is noted a striking reduction both in protein and total ash; but a considerable rise in the fat. Here again the percentage of fat in one specimen (No. 7) was so low as clearly to be an exceptional individual variation. The average for the remaining one individual and three composite samples was 4.37 per cent. The average values for the other elements were sugar 7.74; protein, 1.56; ash, 0.2407 and total solids, 13.39 per cent.

While these figures are too few in number to be regarded as more than suggestive, they indicate that the secretion of woman's milk is richer in fat, in protein, in salts and in total solids in the early weeks of lactation than at a later period. It corresponds also to the needs of the infant during the period of his most rapid growth. This, as one would expect, is most striking in the case of the ash and the protein.

*Mature.*—The figures for mature milk are from seventeen large individual samples—usually the twenty-four hours' secretion. Though the number of cases is small, the figures have a value which does not attach to those obtained from a study of small samples. They were all from healthy women whose infants were thriving; they were all upon a mixed diet. The age and the number of the child are given in the table. As would be expected, the figures show considerable individual variation in all the elements; this is least in the protein and greatest in the fat. The figures for sugar are given both as obtained directly by reduction and as estimated, in the more common way, by difference. Compared with the early or transition period there is noted a fall in the total solids which affects all the elements except the sugar, but is most noteworthy in the protein and the ash.

For comparison we at first divided these into two groups: the first group of nine samples of one to four months' milk; the second of eight samples of four to nine months' milk. But the differences in the individual samples and in the averages of these two periods were so slight that a separate grouping seemed unnecessary.



The individual variations in this period are most marked in the fat, the range being from 1.67 to 5.48 per cent. The smallest variation is seen in the sugar. The lowest value found was 5.54 per cent. Except for this one specimen the range was between 7.05 and 8.45 per cent. obtained by reduction, and 7.11 and 8.36 per cent. by difference. In the main, therefore, the values obtained for sugar by the two methods do not differ greatly. The range for the total protein is from 0.88 to 1.5 per cent.; the figures in all but three of the seventeen samples fall between 1 and 1.3 per cent. The relation of the casein and albumin continues fairly uniform throughout both periods. The range in the total ash is between 0.1590 and 0.2433 per cent., but the greater number of the samples fall between 0.18 and 0.22 per cent.

In the colostrum period the relation of the ash to total protein is 1 : 7; in the transition period it is 1 : 6; throughout the mature period it is about 1 : 5, or the same ratio as exists in cow's milk.

*Late.*—Greater interest attaches to a study of the ten samples of late milk—tenth to twentieth month, for so few observations on milk of this period have been published. There are two or three exceptional findings, but as a group the milks for this period show no constant or essential differences in any of their constituents from those of the mature period. The marked fall in the protein and ash noted by some observers was not regularly seen in our cases. In but two of the ten cases was the protein low, while in five of the ten cases the ash was higher than the average of the mature period.

Excluding the three very exceptional individual variations mentioned, two of fat and one in total ash, occurring in the colostrum and transition periods, the average percentage composition of the milk for each of the different periods is as shown in Table 2.

We have brought together in Table 3 the results of the analyses found in medical literature which bear on the composition of the ash of normal woman's milk. The cases have been grouped according to the division of Schloss to whose work we are much indebted. The total number of analyses we have been able to collect is but twenty-eight, even though we include five which are either incomplete or open to some question.

The results of our own observations on the different salts which make up the ash are given in Table 4, classified according to periods.

The variations in the total ash for the different periods have already been considered. The striking fall in the ash value continues only from the colostrum through the transition period; after this little regular variation is shown by these figures. In the samples in which the total ash was exceptionally low, while there was a reduction in all the ash constituents, it was most marked in the  $\text{Na}_2\text{O}$  and  $\text{K}_2\text{O}$ .

Of all the ash constituents the percentage of  $\text{CaO}$  continues most nearly constant throughout. This is shown not only by periods but by individual samples. In only two of the entire thirty-eight samples analyzed did the value for this element differ very widely from the

TABLE 2.  
*Percentage Composition of Woman's Milk by Periods.*

Period	No. of Analyses	Fat	Sugar	Protein	Casein	Albu- min	Ash	Total Solids
Colostrum, (1-12 da.)	5	2.83	7.59	2.25	...	...	.3077	13.42
Transition, (12-30 da.)	6	4.37	7.74	1.56	...	...	.2407	13.39
Mature, (1-9 mos.)...	17	3.26	7.50	1.15	.43	.72	.2062	12.16
Late, (10-20 mos.)....	10	3.16	7.47	1.07	.32	.75	.1978	12.18

average. Both these (Nos. 15 and 17) were individual samples of three months' milk; these two represent the range of values found, viz., 0.0295 and 0.0702 per cent.

The figures for  $\text{MgO}$  show a wider variation, not only for the different periods, but in the individual samples. The fall from the highest value in the colostrum period to the lowest in the transition period is without evident explanation. The lowest value, 0.0036 per cent., was in an individual sample of transition milk (No. 10), the highest, 0.0161, was in an individual sample in the colostrum period (No. 4).

The figures for  $\text{P}_2\text{O}_5$  like those for  $\text{CaO}$  do not show wide variations either in the different periods or in the separate samples. The values are somewhat higher in the colostrum and transition periods; but they show no regular change till the late period when the smallest average is seen. The range is from 0.0526, an individual colostrum sample (No. 3) to 0.0212, an individual sample of a six months' milk (No. 25) in which also the total ash was the lowest met with in our observations.

TABLE 3.  
Distribution of the Ash—Grams Per 100 C.C. of Milk.  
*A. Colostrum Period.*

No.	Author	Age of Woman, Yrs.	Age of Child, Days	Sample	Amt. in c.c.	Total Ash	CaO	MgO	P <sub>2</sub> O <sub>5</sub>	Na <sub>2</sub> O	K <sub>2</sub> O	Cl	Percentage Composition of the Ash					
													CaO	MgO	P <sub>2</sub> O <sub>5</sub>	Na <sub>2</sub> O	K <sub>2</sub> O	Cl
1	Söldner.....	..	5-8	Composite, 2 women	..	.3497	.0330	.0068	.0330	.0470	.1370	.0710	9.4	1.9	9.6	13.6	39.1	20.4
2	Söldner.....	..	5-9	Composite, 6 women	..	.2894	.0400	.0052	.0330	.0280	.1000	.0660	13.8	1.8	11.6	9.8	34.6	22.2
3	Söldner.....	..	5-9	Composite, 6 women	..	.3382	.0420	.0077	.0280	.0670	.0870	.0920	12.6	1.4	8.4	19.9	25.9	27.1
4	De Lange*	..	4-10	Composite, 10 women	..	..	..	..	..	.0950	.0640	..	..	..	..	..	..	..
	De Lange.....	..	4-10	Composite, 23 women	..	..	.0410	.0090	.0580	..	..	.0680	..	..	..	..	..	..
5	Schloss.....	22	11-13	Individual	..	.3048	.0335	.0069	.0380	.0532	.0795	.0892	11.0	2.2	12.4	17.4	26.0	29.2
6	Birk†.....	..	..	..	..	.2814	.0360	.0093	[.1137]	.0544	.0770	..	12.7	3.3	[40.4]	19.3	27.3	..
	Averages.....	..	..	..	..	.3127	.0375	.0074	.0380	.0574	.0907	.0772	11.9	2.1	10.5	16.0	30.5	24.7

*B. Transition Period.*

7	Söldner.....	..	7-12	Composite, (of several women)	..	.2620	.0350	.0050	.0330	.0360	.0790	.0570	13.5	1.7	12.7	13.7	30.1	21.8
8	Söldner.....	..	22-63	Composite, (of several women)	..	.2185	.0410	.0044	.0320	.0170	.0680	.0360	18.9	2.0	14.5	7.9	31.1	16.2
9	Schloss.....	22	14-17	Individual	760	.2331	.0400	.0069	.0393	.0338	.0678	.0425	17.1	2.9	16.8	14.4	29.0	18.2
10	Schloss.....	22	26	Individual	1,180	.1904	.0355	.0081	.0393	.0192	.0558	.0310	18.6	4.2	20.6	10.1	28.9	16.2
11	Bunge.....	..	15	Individual	..	.2219	.0328	.0064	.0473	.0232	.0780	.0438	14.7	2.9	21.3	10.4	35.1	19.7
12	Bunge.....	..	18	Individual (same)	..	.2187	.0343	.0065	.0469	.0257	.0703	.0445	15.6	2.9	21.4	11.8	32.1	20.3
	Averages.....	..	..	..	..	.2241	.0364	.0062	.0396	.0258	.0698	.0424	16.4	2.7	17.9	11.3	31.0	18.7

TABLE 3.—Continued.  
C. Mature Period.

No.	Author	Age of Woman, Yrs.	Age of Child, Days	Sample	Amt. in c.c.	Total Ash	CaO	MgO	P <sub>2</sub> O <sub>5</sub>	Na <sub>2</sub> O	K <sub>2</sub> O	Cl	Percentage Composition of the Ash					
													CaO	MgO	P <sub>2</sub> O <sub>5</sub>	Na <sub>2</sub> O	K <sub>2</sub> O	Cl
13	Aberghalden	..	Mos.	.....	.....	.....	.0489	.0065	.0585	.0253	.0795	.0468	19.1	2.5	22.9	9.9	31.2	18.3
14	Schloss	21	2 1/4	Individual	2,000	.1886	.0387	.0094	.0431	.0219	.0522	.0255	20.5	4.9	22.8	11.6	27.6	13.3
15	Schloss	27	3	Individual	790	.2454	.0567	.0116	.0501	.0200	.0650	.0425	23.1	4.7	20.4	8.1	26.4	17.3
16	Schloss	19	3 1/4	Individual	1,360	.1848	.0370	.0100	.0342	.0192	.0549	.0308	20.0	5.4	18.5	10.3	29.7	16.7
17	Schloss	27	4 1/4	Individual	1,680	.2088	.0455	.0087	.0416	.0210	.0608	.0411	21.8	4.1	19.9	10.0	29.1	19.7
18	Schloss	..	..	Composite	.....	.1913	.0386	.0076	.0447	.0162	.0529	.....	20.1	3.9	23.3	8.4	27.6	.....
19	Schloss	..	..	Composite	.....	.1838	.0380	.0073	.0383	.0196	.0536	.0312	20.7	3.9	20.8	10.6	29.1	16.9
20	Soldner	..	3 1/4	Individual	.....	.1790	.0350	.0060	.0260	.0180	.0590	.0330	19.4	3.5	14.4	10.1	32.8	18.3
21	Blauberg	..	..	.....	.....	.1988†	.0394	.0068	.0294	.0049	.0690	.0294	19.8	3.4	14.7	2.4	34.7	14.7
22	Pelka	..	..	Composite	.....	.1828	.0474	.....	.0387	.0258	.0848	.0294	25.9	.....	21.1	14.1	46.3	16.0
23	Pelka	..	..	Composite	.....	.2484	.0328	.....	.0450	.0165	.0643	.0244	13.2	.....	18.1	6.6	25.9	9.8
24	Backhaus and Cronheim	..	..	.....	.....	.....	.....	.....	.....	.....	.....	.....	17.4	3.2	14.8	11.9	33.7	15.5
25	Backhaus and Cronheim	..	..	.....	.....	.....	.....	.....	.....	.....	.....	.....	15.5	2.1	11.8	15.9	27.3	23.9
				Averages	.....	.1955	.0416	.0082	.0409	.0189	.0632	.0340	19.5	3.7	18.5	10.3	30.7	17.5

## D. Late Period.

26	Schloss.....	21	II	Individual	1,650	.1476	.0285	.0073	.0349	.0177	.0440	.0228	19.3	4.9	23.6	12.3	29.8	15.4
27	Schloss.....	..	II 1/4	Individual	720	.1508	.0305	.0073	.0376	.0156	.0449	.0255	20.2	4.8	24.9	10.4	29.8	16.9
28	Schloss.....	26	II 1/4	Individual	1,075	.1552	.0280	.0062	.0431	.0162	.0465	.0251	18.0	3.9	27.7	10.4	29.9	16.1
				Averages....	.....	.1512	.0290	.0069	.0385	.0165	.0451	.0245	19.2	4.5	25.4	11.0	29.8	16.1

\* De Lange's work is usually quoted as a single, complete analysis. The original publication shows that part of the ash was determined from one sample, and the other elements from a second.

† Birk's figures are often quoted and hence are introduced. His value for P<sub>2</sub>O<sub>5</sub> differs so widely from other published figures, as well as ours, that we have omitted this case in computing the averages.

‡ By addition, the figure by determination is not given in the original paper.

TABLE 4.  
Distribution of the Ash—Grams Per 100 C.C. of Milk.  
A. Colostrum Period.

No.	Age of Woman, Yrs.	Age of Child, Days	No. of Child	Sample	Total Ash	CaO	MgO	P <sub>2</sub> O <sub>5</sub>	Na <sub>2</sub> O	K <sub>2</sub> O	Cl
1	19-21	3-4	...	Composite, 5 women	.2960	.0431	.0092	.0308	*	*	.0640
2	18-25	3-5	...	Composite, 6 women	.3312	.0527	.0053	.0369	.0453	.0938	.0744
3	.....	5-7	...	Individual	[.1747]	.0476	.0076	.0526	*	*	.0304
4	.....	5-8	...	Individual	.2921	.0415	.0161	.0501	*	*	.0693
5	20-25	5-12	...	Composite, 4 women	.3117	.0382	.0125	.0347	*	*	.0462

## B. Transition Period.

6	16-21	12-30	...	Composite, 4 women	.2335	.0450	.0071	.0497	*	*	.0590
7	32	14	...	Individual	.2132	.0338	.0049	.0308	*	*	.0639
8	20	15-18	...	Composite, 2 women	.2471	.0437	.0073	.0370	.0392	.0585	.0598
9	19-32	14-28	...	Composite, 5 women	.3042	.0498	.0067	.0397	*	*	.0955
10	18	21	...	Individual	.2262	.0389	.0036	.0419	.0136	.0811	.0368
11	19	21	...	Individual, 24 hours	.2204	.0343	.0051	.0433	.0239	.0731	.0334

## C. Mature Period.

12	18	Mos. 1	1	Individual, 48 hours	.1825	.0413	.0052	.0265	.0183	.0480	.0251
13	32	2	2	Individual, 24 hours	.2200	.0497	.0092	.0276	.0131	.0638	.0423
14	24	2	3	Individual, 24 hours	.1902	.0414	.0084	.0317	.0148	.0502	.0364
15	26	3	2	Individual, 36 hours	.1717	.0295	.0057	.0239	.0214	.0494	.0352
16	25	3	2	Individual, 24 hours	.2127	.0459	....	.0431	*	*	.0383
17	23	3	1	Individual, 24 hours	.2135	.0702	.0089	.0409	.0145	.0366	.0378
18	32	3	2	Individual, 24 hours	.2254	.0536	.0106	.0392	.0143	.0618	.0313
19	27	3½	2	Individual, 24 hours	.2433	.0555	.0091	.0440	.0175	.0714	.0378
20	23	3¾	1	Individual, 24 hours	.1914	.0503	.0090	.0310	.0250	.0504	.0318

\* The values for Na and K in the earlier samples have not been included in the table because the method of ashing the dried material, and later of igniting to drive off ammonium salts, was one in which a slight loss of Na and K by volatilization is possible. The Stölte method described elsewhere in the paper was used in the case of most of the other samples.

TABLE 4.—Continued.

## C. Mature Period.

No.	Age of Woman, Yrs.	Age of Child, Mos.	No. of Child	Sample	Total Ash	CaO	MgO	P <sub>2</sub> O <sub>5</sub>	Na <sub>2</sub> O	K <sub>2</sub> O	Cl
21	39	4	2	Individual, 24 hours	.2090	.0456	.0067	.0331	.0129	.0599	.0437
22	23	4	1	Individual, 24 hours	.2141	.0535	.0077	.0366	.0112	.0633	.0313
23	26	5	1	Individual, 24 hours	.2281	.0545	.0072	.0386	.0126	.0661	.0320
24	37	6	2	Individual, 48 hours	.1922	.0412	.0082	.0299	.0158	.0582	.0315
25	30	6	4	Individual, 36 hours	.1590	.0354	.0072	.0212	.0031	.0545	.0304
26	30	7	2	Individual, 24 hours	.2402	.0513	.0084	.0514	.0133	.0644	.0376
27	22	8½	1	Individual, 48 hours	.2158	.0430	.0092	.0380	.0169	.0688	.0444
28	38	9	1	Individual, 2 days	.1967	.0426	.0097	.0301	.0204	.0531	.0358

## D. Late Period.

29	31	10	4	Individual, 4 days	....	....	....	.0281	.0126	.0469	.0439
30	25	10½	1	Individual, 2 days	.2148	.0433	.0097	.0282	.0282	.0490	.0474
31	23	11¼	1	Individual, 2 days	.2107	.0483	.0045	.0348	.0133	.0594	.0405
32	33	12	3	Individual, 3 days	.2141	.0405	.0071	.0332	.0315	.0673	.0570
33	30	12½	4	Individual, ½ day	.1728	.0390	.0059	.0227	.0188	.0504	.0339
34	26	12½	...	Individual, 1 day	.2108	.0320	.0072	.0297	.0338	.0551	.0521
35	..	14	...	Individual, 3 days	.1790	.0396	.0093	.0270	.0134	.0545	.0441
36	30	15	4	Individual, ½ day	.1675	.0348	.0093	.0296	.0146	.0556	.0301
37	..	18	...	Individual, 5 days	.2251	.0461	.0054	.0422	.0151	.0576	.0500
38	35	20	6	Individual, 3 days	.1855	.0280	.0048	.0284	.0135	.0794	.0433

## Averages for the Different Periods.

	No. of Analyses	Total Ash	CaO	MgO	P <sub>2</sub> O <sub>5</sub>	Na <sub>2</sub> O	K <sub>2</sub> O	Cl
Colostrum (1-12 days)....	5	.3077	.0446	.0101	.0410	.0453	.0938	.0568
Transition (12-30 days)...	6	.2407	.0409	.0057	.0404	.0255	.0709	.0580
Early mature (1-4 months)	9	.2056	.0486	.0082	.0342	.0174	.0539	.0351
Middle mature (4-9 months).....	8	.2069	.0458	.0080	.0348	.0132	.0609	.0358
Late milk (10-20 months)...	10	.1978	.0390	.0070	.0304	.0195	.0575	.0442

For reasons already mentioned (see note to Table 4) we have reported only a single figure for  $\text{Na}_2\text{O}$  and  $\text{K}_2\text{O}$  in the colostrum period, and but three in the transition period. These figures indicate the highest value for  $\text{Na}_2\text{O}$  in the colostrum period, next in the transition period, while during the early and latter part of the mature period it falls to less than one-third the amount. It is noteworthy that three of the ten late milks show a very high sodium figure; the figures for the other samples correspond with those of the mature period. The extreme individual variations of sodium are considerable in the mature period—from 0.0031 (No. 25) to 0.0250 (No. 20); but in the remaining cases of this period the value for this constituent is very close to the average.

Like  $\text{Na}_2\text{O}$  the value for  $\text{K}_2\text{O}$  is highest in the colostrum period and next in the transition period, but after this time it is quite uniform even in the late period. The individual variations are smaller than in the case of the sodium.

The figures for Cl vary much like the two constituents just mentioned; in all periods they bear a very close relation to the combined values of  $\text{Na}_2\text{O}$  and  $\text{K}_2\text{O}$ , indicating that it is combined as chlorids of sodium and potassium in the milk. The  $\text{P}_2\text{O}_5$  and the CaO have also a close relationship in all periods, indicating their existence in milk as calcium phosphate.

Considering the milk by periods, we note that the high ash of the colostrum period is chiefly due to the higher values for  $\text{Na}_2\text{O}$  and  $\text{K}_2\text{O}$ ; the values for CaO and  $\text{P}_2\text{O}_5$  differing but slightly from those of the later periods. The values of the transition period show a further fall in  $\text{Na}_2\text{O}$  and  $\text{K}_2\text{O}$  and Cl, but little change in the other elements.

No constant or essential differences are seen in the values for the salts which make up the ash in either the early or latter part of the mature period, nor, in fact, even in the late period. These were grouped separately for the purpose of determining whether such a difference existed. Even in the late period the values show very little difference from those in the preceding period.

Some details of the women furnishing the late milks are interesting. In four instances (No. 29, 33, 34 and 36) the supply was still abundant and nursing was being carried on successfully (Nos. 33

TABLE 5.  
Percentage Composition of the Ash.  
*A. Colostrum Period.*

No.	Age of Woman, Yrs.	Age of Child, Days	No. of Child	Sample	Total Ash	CaO	MgO	P <sub>2</sub> O <sub>5</sub>	Na <sub>2</sub> O	K <sub>2</sub> O	Cl
1	19-21	3-4	...	Composite, 5 women	.2960	14.6	3.1	10.4	....	....	21.6
2	18-25	3-5	...	Composite	.3312	15.9	1.6	11.2	13.7	28.1	22.5
3	....	5-7	...	Individual	.1747*	27.2	4.4	30.1	....	....	17.4
4	....	5-8	...	Individual	.2921	14.2	5.5	17.2	....	....	23.7
5	20-25	5-12	...	Composite, 4 women	.3117	12.3	4.0	11.1	....	....	14.8

*B. Transition Period.*

6	16-21	12-30	...	Composite, 4 women	.2335	19.3	3.0	21.3	....	....	25.3
7	32	14	...	Individual	.2132	15.9	2.3	14.4	....	....	30.0
8	20	15-18	...	Composite, 2 women	.2471	17.7	3.0	15.0	15.9	23.5	24.2
9	19-32	14-28	...	Composite, 5 women	.3042	16.4	2.2	13.1	....	....	31.4
10	18	21	...	Individual	.2262	17.2	1.6	18.5	6.0	35.8	16.3
11	19	21	...	Individual, 24 hours	.2204	15.6	2.3	19.6	10.8	33.2	15.1

*C. Mature Period.*

		Mos.									
12	18	1	1	Individual, 48 hours	.1825	22.6	2.8	14.5	10.0	26.3	13.8
13	32	2	2	Individual, 24 hours	.2200	22.6	4.2	12.6	6.0	29.0	19.0
14	24	2	3	Individual, 24 hours	.1902	21.8	4.4	16.7	7.8	26.4	19.2
15	26	3	2	Individual, 36 hours	.1717	17.2	3.3	13.9	12.5	28.8	20.5
16	25	3	2	Individual, 24 hours	.2127	21.6	1.3	20.2	....	31.9	18.0
17	23	3	1	Individual, 24 hours	.2135	32.9	4.2	19.2	6.8	17.1	17.7
18	32	3	2	Individual, 24 hours	.2254	23.8	4.7	17.4	6.3	27.4	13.9
19	27	3½	1	Individual, 24 hours	.2433	22.8	3.7	18.1	7.2	29.4	15.5
20	23	3¾	2	Individual, 24 hours	.1914	26.3	4.7	16.2	13.1	26.3	16.6
21	39	4	1	Individual, 24 hours	.2090	21.8	3.2	15.8	6.2	28.7	20.9

\* This is so exceptional a figure that the values are excluded in computing the averages.



TABLE 5.—Continued.

No.	Age of Woman, Yrs.	Age of Child, Mos.	No. of Child	Sample	Total Ash	CaO	MgO	P <sub>2</sub> O <sub>5</sub>	Na <sub>2</sub> O	K <sub>2</sub> O	Cl
22	23	4	1	Individual, 24 hours	.2141	25.0	3.6	17.1	5.2	29.6	14.6
23	26	5	2	Individual, 24 hours	.2281	23.9	3.2	16.9	5.5	29.0	14.0
24	37	6	4	Individual, 48 hours	.1922	21.4	4.3	15.6	8.2	30.3	16.4
25	30	6	2	Individual, 36 hours	.1590	22.3	4.5	13.3	2.0	34.3	19.1
26	30	7	1	Individual, 24 hours	.2402	21.4	3.5	21.4	5.5	26.8	15.7
27	22	8½	1	Individual, 48 hours	.2158	19.9	4.3	17.6	7.8	31.8	20.6
28	28	9	...	Individual, 2 days	.1967	21.6	4.9	15.3	10.4	27.0	18.2

*D. Late Period.*

29	31	10	4	Individual, 4 days	†						
30	25	10½	1	Individual, 2 days	.2148	20.2	4.5	13.1	13.1	22.8	22.1
31	23	11¼	1	Individual, 2 days	.2107	22.9	2.1	16.5	6.3	28.2	19.2
32	33	12	3	Individual, 3 days	.2141	18.9	3.3	15.5	14.7	31.4	26.6
33	30	12½	4	Individual, ½ day	.1728	22.6	3.4	13.2	10.9	29.2	19.6
34	26	12½	...	Individual, 1 day	.2108	15.2	3.4	14.1	16.1	26.2	24.8
35	..	14	...	Individual, 3 days	.1790	22.1	5.2	15.1	7.5	30.4	24.6
36	30	15	4	Individual, ½ day	.1675	20.8	5.6	17.7	8.7	33.2	18.0
37	..	18	...	Individual, 5 days	.2251	20.4	2.4	18.8	6.7	25.6	22.2
38	35	20	6	Individual, 3 days	.1855	15.1	2.6	15.3	7.3	42.8	23.4

† Percentage could not be calculated as total ash was not determined.

*Average Percentage Composition of Ash for the Different Periods.*

	CaO	MgO	P <sub>2</sub> O <sub>5</sub>	Na <sub>2</sub> O	K <sub>2</sub> O	Cl
Colostrum.....	14.2	3.5	12.5	13.7	28.1	20.6
Transition.....	17.0	2.4	16.9	10.9	30.8	23.7
Mature.....	22.9	3.7	16.6	7.5	28.3	17.3
Late.....	19.8	3.6	15.5	10.1	30.0	22.3

and 36 were from the same woman). Three samples (Nos. 30, 35, 38) were taken at the very end of a previously successful lactation, two or three days' pumping being usually required to obtain sufficient

milk for analysis. In three cases (Nos. 31, 32, 37) there was still a good supply of milk, the infants being partly nursed and partly fed.

From a practical standpoint the best idea of the salts of woman's milk is obtained from a study of the percentage composition of the ash. This is given in the Table 5.

A comparison of the percentage composition of the salts of woman's milk, mature period, with those of cow's milk is highly instructive. The figures for cow's milk are the averages of nine analyses of our own, made of milk from mixed herds.

TABLE 6.

*Comparison of the Percentage Composition of the Ash of Woman's and Cow's Milk.*

	CaO	MgO	P <sub>2</sub> O <sub>5</sub>	Na <sub>2</sub> O	K <sub>2</sub> O	Cl
Mature woman's milk .	23.3	3.7	16.6	7.2	28.3	16.5
Cow's milk . . . . .	23.5	2.8	26.5	7.2	24.9	13.6

The close correspondence between these figures is very striking. In all the constituents except P<sub>2</sub>O<sub>5</sub>, the percentages of the different salts in the two milks are practically the same. The higher proportion of phosphorus in cow's milk is due to the large amount in the casein. Though the *proportions* of the different salts of the ash in cow's milk are so nearly those of woman's milk, the *amount* in cow's milk is about three and a half times as great. Unless, therefore, cow's milk has been diluted with more than twice its volume, the amount of these inorganic constituents furnished to the infant is equal to that which he receives in woman's milk. The addition of lime or other inorganic salts to cow's milk because they are lacking in amount is therefore quite unnecessary in infant feeding.

A general comparison of the results of our analyses with those of the other authors cited shows a general agreement in most of the essential points. There is, however, less variation between the findings in our individual cases than between the findings of the different investigators who have made but a small number of analyses. It is not unlikely that differences in methods or in technic may be responsible, in part, at least, for some of these wider variations. We feel

that enough analyses of the salts of normal woman's milk have now been made to afford a basis for comparison with abnormal milks studied in connection with metabolism observations.

There remains for brief discussion a consideration of the iron content of milk. For reasons already given, we have not undertaken a study of this part of the subject. The results of the analyses of the authors quoted in the earlier part of the paper indicate that the figures previously given for iron are too high; that woman's milk contains but 1.7 mg. of iron in a liter, while cow's milk has barely one-third as much—really a negligible quantity. By these figures iron forms but 0.00007 per cent. of the ash of cow's milk, and 0.00015 per cent. of the ash of woman's milk.

#### SUMMARY.

1. The use of large individual samples of milk for analyses has advantages not offered by such small ones as have been commonly employed. For a determination of the inorganic constituents large samples are indispensable.

2. In the colostrum period woman's milk has high protein and high ash with rather low fat; in the transition period the protein and ash are lower while the fat is higher; in the mature period (after one month) the composition of normal milk does not vary in any essential or constant way quite up to the end of lactation. The only striking feature of late milk is a decline in quantity, though there is noted a slight fall in all the solid constituents except the sugar.

3. Of the different constituents of milk the least variation both in individuals and in periods is seen in the sugar. The proportion of this is somewhat higher than the generally accepted 7 per cent.; 7.5 per cent. is nearer the correct figure.

4. The greatest individual variations are seen in the fat, though the period variations in fat are not marked.

5. The protein is highest in the colostrum period and falls to a little over half the proportion in mature milk, during which period it is seldom over 1.25 per cent.; of this about one-third is casein, and two-thirds lactalbumin.

6. The high ash of the colostrum period is chiefly due to the amount of  $\text{Na}_2\text{O}$  and  $\text{K}_2\text{O}$ . Of the salts which make up the ash,

the greatest individual, as well as the greatest period, variations are seen in the  $\text{Na}_2\text{O}$ ; the least individual and period variations are seen in the  $\text{CaO}$ , the proportion of which is nearly constant throughout the period of lactation.

7. The largest constituent of the ash of woman's milk is  $\text{K}_2\text{O}$ ; this with the  $\text{CaO}$  together make up more than half the total ash.

8. Although in amount the total ash of cow's milk is about three and one-half times as great as that of woman's milk, the proportion of different salts which make up the ash is nearly the same, the only exceptions being that cow's milk has more  $\text{P}_2\text{O}_5$  and less iron.

We desire to express our thanks to those who have assisted us in obtaining specimens of milk for examination; especially to Dr. Herman Schwarz, Prof. J. Clifton Edgar, Dr. F. C. Freed and to the House Staff of the New York Foundling Hospital.

## CLINICAL AND ELECTROCARDIOGRAPHIC STUDIES ON THE ACTION OF DIGITALIS.\*

By ALFRED E. COHN, M.D.

*(From the Hospital of The Rockefeller Institute for Medical Research.)*

It is extraordinary that, after continuous use for 130 years, the study of the action of digitalis retains so vital an interest, not only in clinical medicine, but also in experimental pharmacology. This interest has been quickened by the fundamental investigations of James Mackenzie on the mechanism of the heart beat and by the construction by Einthoven of a galvanometer suitable for registering the action currents of the heart.

I mention James Mackenzie especially, because in the bewildering variety of studies which he has stimulated he has, almost alone, kept in view the essential objects of investigation in the domain of cardiovascular disease. These are, first, the study of symptoms suggesting the presence of incipient heart disease, and second, the study of the mechanism of heart failure and its treatment. His study of the disturbed rhythms of the heart was undertaken to ascertain whether they had a vital relation to these subjects, and to learn whether an appreciation of their significance would aid in understanding altered function.

I doubt very much whether the disordered rhythms of the heart, taken by themselves, presented a subject of fundamental interest to Mackenzie, and in that, too, he has differed from his followers. The reader remembers, no doubt, that he called his book "Diseases of the Heart;" but its theme may be said to be the arrhythmias in their relation to heart failure. This view of the arrhythmias gives them a new significance in studying the action of drugs. They can

\* Read before the Section on General Medicine, College of Physicians, Philadelphia, Dec. 14, 1914.

be made to serve as important indicators in estimating the value of therapeutic effects. Mackenzie's work, therefore, provides an important reason for reexamining the action of digitalis. Just as it was important to recognize the arrhythmias and to appreciate their significance, it was necessary to have far more accurate and more satisfactory methods of registering the motions of the heart than arterial and venous tracings, which record only the motions of the vessel walls, and in many instances record them badly or not at all. The string galvanometer, although it is not a substitute for them, has come to replace the older methods by one eminently practical, free from error due to the personal equation and reliable within the limits of its capacity. This instrument has made Mackenzie's work more easily available. Its use, as I hope to show, has made possible the introduction of added criteria for recognizing the action of drugs. Mackenzie's new conceptions and Einthoven's new instrument, reported almost simultaneously, made therapeutic investigation from a new point of view desirable.

I want to discuss the effects of digitalis on heart rate, on conduction, on the electrocardiogram, on blood pressure and on diuresis. But because there is still a very distinct divergence of opinion between practitioners of clinical medicine and pharmacologists, as to how digitalis acts, I must take account of the views of both in presenting the subject. But the matter is not simple, for clinicians differ in the results of their observations, and pharmacologists on the interpretation of experiments.

The school of Schmiedeberg still maintains that the main action of a digitalis body is on heart muscle; the school of Gottlieb, while it does not deny to digitalis bodies a cardiac action, still maintains the view that it has an important action on the walls of the arteries. Both schools find that the drug increases the excursion of the heart in contraction, both believe that it elevates blood pressure, both believe that it increases the amount of renal secretion.

It is perhaps not an overstatement to say that in a general way clinicians have been too much influenced by these experimental results and have felt obliged to find that the administration of the drug in patients results in parallel phenomena. It requires a very small experience in treating patients suffering from heart disease to find

oneself disappointed because the expected results did not occur. And when discrepancies were noticed, the discovery was not often followed by an effort to explain them. The subject was often dismissed by finding fault with the potency of the drug or by discovering an idiosyncrasy in the patient. But even if drugs were always potent and there were no individual idiosyncrasies, it is extremely likely that patients would continue to react in different manners to the drug. And the reason for that must be that individuals, although they suffer from what in a general sense is called heart disease, yet present a great variety of clinical pictures.

If the cardinal symptoms of heart failure alone are considered, rapid pulse, disturbed rhythm, high blood pressure, dyspnea, pain and edema, it becomes clear that patients do not usually present all of these signs at once, that some present one or two of them, or a group. It must be apparent that the pharmacologic experiment does not take this diversity of circumstances into consideration; the experiment is done under artificial conditions, and the doses which are employed bear no relation to those permitted in clinical medicine. There can be no question of the usefulness to therapeutics of these experiments; as guides, they are indispensable, but it must be clear that they neither replace nor parallel the clinical conditions we must treat. That there has consequently been a divergence between the results of pharmacologists and clinicians in a practical sense was inevitable. The responsibility for it is probably shared equally by both. Pharmacologists have dealt usually with simple normal conditions; clinicians with complex pathologic ones.

In therapeutics, divergent accounts of the action of the digitalis bodies are also found. For instance, they are sometimes said to reduce the heart rate, and to act almost as specifics; others deny such an action. In the presence of fever their use is discouraged by some as valueless. In the domain of valvular defects a variety of indications and contradictions for giving the drug prevails; some regard these as unimportant. Until recently, some avoided it in cases of high systolic arterial pressure, because it was reputed to elevate this to dangerous limits. Some, again, have detected a striking effect in a short time (Marvin), while others have recently reported that it has no effect on pressure at all. Its effect on diuresis has, on the whole,

been commonly held and has been gained on account of the disappearance of edema, when this sign of heart failure is present.

A drug having apparently such very powerful actions as digitalis must be extremely valuable and must gain in usefulness if its modes of action are carefully studied and precisely recorded. It has been no easy task to reconcile the divergent views drawn from experimental experience, and equally difficult to understand the contradictions expressed by reliable clinicians. We have therefore been engaged in the Hospital of the Rockefeller Institute in the attempt to understand and to reconcile these differences, in order to make more available the data of experimental pharmacology, and to ascertain

*Classification of Patients.*

A. Normal rhythm .....	a. Without edema	1. With normal blood pressure
	b. With edema	2. With high blood pressure
B. Auricular fibrillation ...	a. Without edema	3. With normal blood pressure
		4. With high blood pressure
	b. With edema	5. With normal blood pressure
		6. With high blood pressure
		7. With normal blood pressure
		8. With high blood pressure

whether the indications for the use of digitalis could be simplified if patients were grouped with a view of delimiting the conditions in which uniform results might be expected. For these purposes it has been deemed wise to study digitalis in the human subject, because in the first place it is about its action in patients that information is wanted, because, in the second, that information can, in practically all respects, under present conditions be satisfactorily obtained, and because, in the third place, the study can be made not only without any prejudice to the patient, but, indeed to his advantage.

It is apparent, if the cardinal symptoms of heart disease during heart failure are considered, rapid pulse, disturbed rhythm, high blood pressure, dyspnea, pain and edema, that not all patients present all of these complaints, and that each patient suffers from only a selection of them; of these symptoms the most obvious basis for the arrangement of patients into groups for the purposes of treatment, especially at the present time, is rhythm. Rhythm, it will be remembered, was the subject with which Mackenzie started. The



basis has, in point of fact, been found extremely serviceable, for in a general way it has been said that the drug acts on cases of auricular fibrillation very successfully, and has even been called, erroneously of course, a specific; while in cases presenting a sinus rhythm it has been said to fail. We have therefore divided patients primarily into fibrillators and nonfibrillators. On the question of rhythm, pulse rate also depends, and we regard these two factors together, but not, of course, as the same. The second basis on which patients can be divided is the question of edema and the retention of water, involving the question of salt metabolism, and these two symptoms may also be regarded as phases of a similar disturbance. The third basis on which we have found it useful to study patients has been on the basis of the height of blood pressure. We do not, of course, pretend that a classification such as this presents all the points of view from which either heart failure or the treatment of heart failure can be viewed; it omits important symptoms such as dyspnea and pain. Often these are cardinal, often they are secondary signs. But, after much consideration, we have adopted it as a useful working plan for testing the effect of drugs. The classification then, is as in the accompanying tabulation.

The group of patients which presents the simplest form of disease is the first one, in which the rhythm of the heart is normal, there is no edema and the blood pressure is not elevated. This is the state found in a patient who, if he presents himself for treatment at all, is in an early stage. It is naturally understood that an early stage of heart failure does not by any means suppose an early stage in heart disease. It is, on the whole, the simplest kind of heart failure which one is called on to treat. The general physiology of such a patient presents the nearest approach to the normal. It is the effect of giving digitalis to him to which we have confined our attention and with which this report principally deals. It is clear that often he requires no drugs; if digitalis is given him it is important to determine what effects are to be expected, and also how the expected effects are to be recognized. This is the method of experimental pharmacology, and the results obtained in this way can be most nearly compared with experimental conclusions. They can be made also to serve as a basis for considering the effects on the more complicated groups.

I have mentioned earlier the topics I mean to discuss, but it is well to dwell for a moment at this point on another. Aside from changes in the physical signs which some clinicians believe themselves capable of appreciating, we have no means whatever of estimating alterations in the functional efficiency of the heart, referable *to the heart itself*. If it were possible, an accurate estimation of contractile force would be the most important single measure to take, but for this we have no satisfactory criteria. Observations on blood flow, blood pressure, estimations of volume output, electrocardiography, have all been equally disappointing. Those effects reported earlier, of changes in the magnitude of ventricular contractions gained in experiments, are more recently admitted (Joseph) to have been obtained by doses far too great. The much smaller doses now used are still much larger than are permitted in therapeutics, but even these fail to show marked changes in the extent of the excursion of the ventricular wall which were formerly held to indicate the nature of effective digitalization. The methods employed in pharmacology are not superior to those now available in clinical medicine. Both are on a par in respect to obtaining objective records of this phase of digitalis action. We must believe, therefore, that if digitalis increases the ability of the ventricles to pump blood, it does so by means of a change which is more subtle than can be distinguished by our methods.

The most important alteration we look for traditionally is in the pulse, or better, the heart rate. This is the first subject we have investigated. For the present we omit fibrillators from consideration. The reduction in the rate of the heart is an effect of digitalis on which we have been taught to rely, both by pharmacologists and clinicians. It is, unfortunately, an effect which we have not found constant in the type of case we are considering. We know also that it fails to reduce rate in the tachycardias of a paroxysmal nature, in those of exophthalmic goiter, and in those of fever. That the pharmacologist could report such an effect depended, no doubt, on the size of his dose. That the clinician believed it to occur depended until recently on his not distinguishing between fibrillators and non-fibrillators. The brilliant reduction in rate was found in the former. It was also found in the sinus rhythm when edema was present; but

neither group of patients is at present under discussion. In the absence of edema, a reduction in rate may be said to occur principally in the hypodynamic and unstable heart, in the heart which for unknown reasons undergoes spontaneous alterations in rate. In hearts of this nature, a natural tendency to reduction is accentuated by administering digitalis. Aside from these, the majority of individuals do not respond to therapeutic doses of digitalis by a fall in heart rate. When amounts equal to 0.4 gm. of leaves a day are given for five to seven days, ventricular slowing does occur, but it occurs as the result of the blocking of the auricular impulses. The latter continue at an unreduced speed and do, as a matter of fact, usually exceed their initial rate. In view of the continued initial, or, in most cases, accelerated auricular rate, it is doubtful whether one may speak of a digitalis slowing of the heart as a primary phenomenon. It depends obviously on the disturbance of a function other than that of stimulus production. We have been led to conclude from our observations, therefore, that digitalis slows the sinus rhythm only in the group of hypodynamic hearts, and that to produce slowing is not a primary function of digitalis in therapeutic doses.

Mention has been made of the blocking of auricular impulses after a sufficiency of digitalis has been given. It is a phase of its action to which Mackenzie drew attention clinically and von Tabora in experiments. Mackenzie, and more especially Lewis, believed it to be produced only when the conducting fibers between auricles and ventricles were previously damaged, more especially by rheumatic inflammation, as evidenced by the fact that the auriculoventricular interval was unduly lengthened. We have been able to show that an action on conduction can invariably be observed in patients, varying from simple lengthening to block. An effect on conduction may therefore be set down as a usual effect of giving the drug, apart from specific preexisting injury. An evidence of delayed conduction can be observed very early after the administration of the drug has been started. We have noticed it within forty-eight hours. In many instances, the auriculoventricular interval gradually lengthened during the succeeding three to five days until partial heart block occurred. We have believed it possible to maintain conduction time

at any length we have thought suitable, by controlling electrocardiographically the amounts of digitalis requisite to produce the effect desired. Not all cases, however, show the gradual lengthening in conduction which I have described; block then occurs with extreme abruptness within a few hours. After the administration of digitalis has been terminated, alterations in conduction usually continue about two days. In the exceptional case, it may persist for two weeks. If gastro-intestinal symptoms have occurred, they do not persist beyond two or three days.

The occurrence of delayed conduction has been dwelt on for the purpose of showing that in certain cases, probably in a large number, the fact that digitalis is acting on the heart can be ascertained early in the administration. When the sign is obtained, it is definite. The problem which arises for decision is whether or not it is a sign which indicates a beneficial action on the heart. For a definite answer on the basis of this sign alone, more experience is required. A greater degree of digitalization can be obtained; but we may learn that on obtaining this effect, the maximum which is desirable has been reached.

When full doses of digitalis have been given, sufficient to cause delayed conduction, abnormal heart rhythms are commonly seen. They have been much described and occur as exaggerations of the normal sinus arrhythmia, extrasystoles, partial heart block, and rarely auricular fibrillation. We shall omit further mention of these. One arrhythmia which is still practically unknown as the result of giving digitalis may be described here. In this form none of the auricular beats is blocked; auricles and ventricles both continue to beat and do so almost at the same rate (Fig. 1). But there is incomplete coordination between them, so that a fixed relation ceases to exist. They must, however, be closely related. The conclusion cannot be escaped that they have an origin in common, or two related origins. If it is a common origin, the lack of exact coordination must depend on a shifting of the site of stimulus production, similar to one we have described in the ventricles, possibly complicated by changes in conduction; if it depends on two competing stimulus-producing foci, they must be actuated by a common factor. It is a matter of interest that we have found arrhythmias of this form

associated only with various forms of intoxicants, notably digitalis. Robinson and Auer have seen it also in the presence of anaphylactic shock.

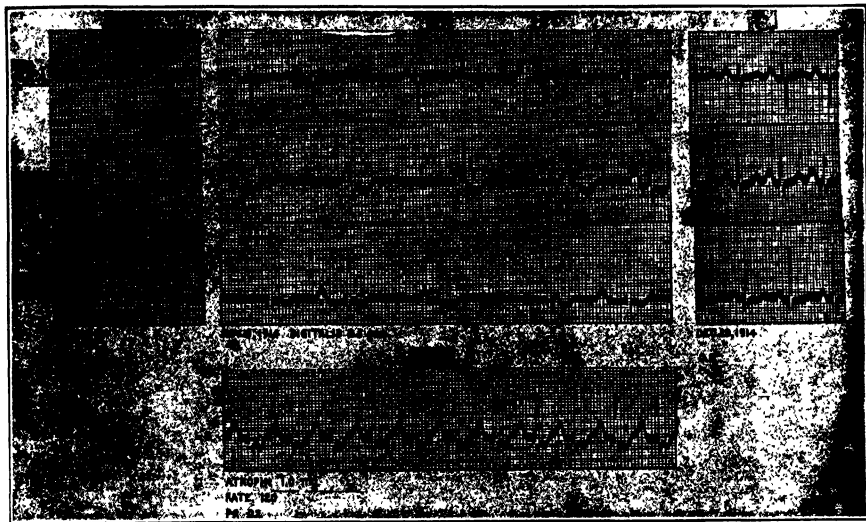


FIG. 1.—Divisions of the ordinates equal 0.1 millivolt; divisions of the abscissae equal 0.04 second. *A*, before digitalis was given; three leads (Einthoven) are shown. *B*, after 2.5 gm. of digitalis had been given; inversion of the *T* wave is shown in Leads 2 and 3; the curves in Leads 1 and 2 show a type of nodal rhythm described in the text. *B* 1 was taken a few minutes after *B*; atropin, 1.0 mg. was injected; it shows that the rhythm of the heart returned to normal; the inverted *T* wave persisted; Lead 3 is shown. *C* shows the three leads; the curves were taken when digitalis had been completely eliminated and the curve had returned to the outline of the control, *A*.

Aside from the lengthening in auriculoventricular conduction which, as has been said, begins in many cases soon after digitalis treatment is initiated and can be taken as an indication that an effect on the heart is being obtained, even though no change in rate has occurred, another new sign in the electrocardiogram appears to be one of great importance. This sign is an alteration in the size, shape, and direction in the *T* wave (Fig. 2, *A* and *B*). If digitalis is given to a patient whose electrocardiogram contains a *T* wave pointing in the usual upward direction, the first change noticed is usually a diminution of the height of this wave, usually in the third

lead first, and later in the second. The first lead may also show changes, but relatively speaking, less frequently. The change may be noticed as early as twenty-four hours to consist in a diminution in the height of the wave, but at the end of forty-eight hours this change is more distinct and the wave may by this time have become iso-electric. At this time or, more often, later, it becomes inverted.

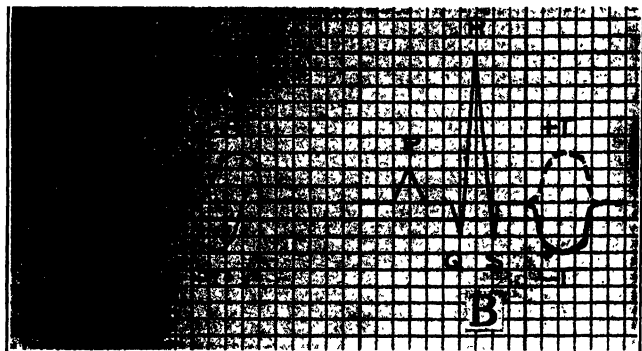


Fig. 2.—*A*. The solid line represents the normal outline of the electrocardiogram. The *T* wave is directed upward. The dotted line shows the change which occurs in the *T* wave under the influence of digitalis. *B*, in a similar way, shows the change in certain cases when the *T* wave is initially directed downward. Under the influence of digitalis it turns upward.

Instead of becoming iso-electric or inverted, it may become diphasic, and in a number of cases of this sort it is easy to distinguish *T* waves consisting of two parts, a first portion which becomes inverted and then returns quickly to a second portion which represents the end of the original wave. The diagram (Fig. 2 *A*) explains this relation. The dotted line represents the altered portion of the curve. There are, of course, many electrocardiograms in which, for pathologic reasons, the *T* wave is originally pointed in a downward direction. If digitalis is given to such patients, the direction of the *T* wave is altered, and during the action of the drug may be directed upward (Fig. 2 *B*). An especially beautiful example of this variety we have found in a case of complete heart block. Usually the negative *T* wave becomes deeper, especially in the third lead. That the change is due to digitalis is proved by the fact that when the drug

is stopped, the *T* wave returns to its original size and shape. The length of time required for it to return to its initial condition varies between five and twenty-two days. To be certain of the change, we have repeated the observation a number of times in the same individual, and to be certain, also, that it is not dependent on a pathologic heart, we have shown it to exist in persons whose hearts are quite normal. It is a sign, then, that the heart is digitalized, and it is a sign which appears early. It appears at a time when no change in conduction need have occurred. It appears in the absence of any alteration in rate. The factors on which the change in the *T* wave depend, we cannot explain precisely at the present time. That it is an evidence of an alteration in the contractile substance of the heart is an inference which is naturally drawn from the circumstance that at the present time the electrocardiogram is generally regarded as the expression of changes in the electric state of the muscle. Whether, in a clinical sense, the change in the *T* wave is to be taken as an indication of beneficial or not beneficial action, it is impossible to say, though there seems little reason to question the fact that it is.

Many matters for consideration arise in relation to the sign. It is a matter of discussion just now, for instance, whether the effect of digitalis on heart muscle depends on an intimate combination of the drug with the muscle (*Speicherung*) in which digitalis is destroyed or consumed, or whether it acts merely by virtue of its concentration in the circulating fluid. In the latter case, the signs of its effect disappear promptly with the withdrawal of the drug, with the diminution in its concentration. The drug is not consumed and, if the solution is recovered, may be used with equal efficiency in a succeeding heart. The essential point of difference is as to whether or not the drug is actually consumed when it is given. In both cases it must, of course, be excreted. If the drug acts merely by virtue of its concentration, a rapid excretion may be expected, and its composition may, of course, be altered; it need not necessarily reappear as the original substance. In the case of its being consumed, its composition on excretion would naturally be altered, but one would expect the rate of its excretion to be much delayed.

In view of what has been said of the change in the *T* wave, under the influence of digitalis, and more especially of the dependence of

the change on alteration in the heart muscle, it is difficult to escape the conclusion that a degree of great intimacy in the combination between the drug and the muscle must have taken place. It is clear that the influence on the muscle may outlast the presence of the drug, but it is difficult to believe that an action of such prolonged duration (three weeks), after giving the drug has ceased, can depend on a simple matter of concentration.

Two criteria have, then, been shown in electrocardiograms to indicate surely the fact that digitalis is acting on the heart; the first in the slight but definite lengthening in conduction, the second the very curious and very constant alteration in the *T* wave. Even if no slowing whatever of the heart occurs, we may be certain, by virtue of finding one or both these phenomena in electrocardiograms, that definite digitalis action on the heart has taken place. Rate, the criterion for which we looked and on which we relied, fails us, but in its place we have these other and surer ones. If these statements prove to be correct, they necessitate an alteration in the indications for the use of digitalis. It is not a drug, at least in the cases we are considering, which alters the rate of the heart—it is a drug which by virtue of its combination with heart muscle, has a bearing on contraction, an effect which is evidenced by its effect on the *T* wave. It appears, then, that the groups of cases in which the drug was supposed to be of value have decreased in number; that is to say, those in which an effect on rate was expected, while groups in which new indications for the use of the drug may be found in the light of muscular and conduction effects we may hope will increase. In consequence, many patients with heart disease from whom digitalis has been withheld may, with new criteria, be drawn into groups for whom it is indicated.

In contrast with the action of digitalis in nonfebrile patients, it is worth referring to the well-known and often mentioned failure of the action of digitalis in cases of fever. Its general failure has been dwelt on by Mackenzie, whose opinions have recently suggested an investigation of the problem to Gunn in Cushny's laboratory. Its failure in pneumonia, in particular, has been dwelt on by G. A. Gibson of Edinburgh. Similar views in regard to the reputed impotence of the drug are no doubt held in this country. Elsewhere, as in Krehl's



clinic, it is usually given and thought to be beneficial. It is not exactly clear what is meant by a failure of digitalis to act in the presence of fever. As an antiphlogistic it need not be considered. What is usually wanted in fever is an agent either to slow the pulse or to increase its strength. It is no doubt a general experience that digitalis does not slow the pulse in fever, and its failure to do so is the complaint most often made. That failure in this respect is not unexpected is clear from our experience in nonfebrile cases. Whether it strengthens the pulse or, in other words, increases the force of ventricular contractions, is an effect which has also been much discussed. Satisfactory objective measures of this are not available, but there is much clinical, that is to say, subjective evidence on the part of observers favoring the idea that it exists. In the absence of evidence bearing on contraction, electrocardiographic evidence can be supplied to show that digitalis has an action during fever of a nature precisely similar to that already described in nonfebrile patients. This evidence consists, as in the nonfebrile, in its influence on the auriculoventricular interval and on the *T* wave. Here, as in the nonfebrile cases, the changes begin within from twenty-four to thirty-six hours and persist for days after defervescence and after the giving of the drug has been stopped. Whatever its nature, it is clear that the same sort of support can be given the heart by digitalis during fever as in its absence. That this is the sort of support that the heart demands may be open to question. It is an error, therefore, to regard its action as a failure until its efficiency from actual, rather than from incorrect points of view, shall have been examined. Possibly as an aid to contraction, much may be gained from its use; but the expectation of obtaining an action on heart rate must be abandoned.

There is, however, a group of febrile cases in which digitalis acts efficiently on rate. In our experience these have not been patients exhibiting the normal sinus rhythm, but patients showing auricular fibrillation or auricular flutter. These rhythms appear spontaneously during attacks of fever and disappear spontaneously afterward. Occasionally the ventricular rate in such cases is inordinately high, the high rate itself being a predisposing factor toward heart failure. The most desirable thing to accomplish in these instances is a reduc-

tion in rate. By the use of intravenous doses of strophanthin, one might expect to block the greater number of auricular impulses which cause the acceleration of the pulse and to reduce the ventricular rate to reasonable limits. In several patients in whom the abnormal rhythms were found, strophanthin was injected and has acted in the described way. Under these circumstances this method of treatment has seemed to us a life-saving remedy. It may be mentioned that, except for its greater speed in action, strophanthin is not necessary and that exactly similar results in cases of flutter have been obtained by the use of digitalis by mouth. The effect on rate which digitalis has on auricular fibrillation and flutter, when these occur in fever, is precisely the same as in the nonfebrile state, and the results obtained are precisely the same. We are led to believe, therefore, although we have reached no definite conclusion, that there is no difference in principle between the way digitalis acts in fever and in nonfebrile conditions. One might venture the suggestion that, from the point of view of heart rate, and from the point of view of an emergency, the possession, in certain cases, of a heart beating after the manner of auricular fibrillation or flutter may, in fever, be a valuable asset, for it is in these rhythms that digitalis, by blocking auricular impulses, is able to slow the pulse rate. It may be mentioned, incidentally, that fever patients having auricular fibrillation initially are not necessarily prejudiced in the outcome of the disease by the abnormal rhythm.

Another topic long a subject of conflict is the relation of digitalis action to blood pressure. Clinicians have often withheld the drug from patients having high pressure in the fear of producing unpleasant consequences, as, for instance, cerebral apoplexy. One of the most recent contributions to this phase of the action of digitalis has been Marvin's. His investigations with a single dose of the tincture in normal medical students led him to conclude that, after five hours, an elevation of from 10 to 15 mm. Hg occurred in systolic pressure, and that the effect of this persisted for fifty hours, when the curve resumed its initial height. Many exact studies have been made in recent years, notably by Mackenzie, Price, and Lawrence. These and other investigators have failed, on the whole, to find pressure changes resulting from the administration of the

drug. The form of this statement demands a certain modification. The clinical reports bear the defects mentioned earlier—that they are based on cases indiscriminately selected, or not grouped at all. They include cases with high pressure and cases with low. The heart mechanism, in itself an important factor, is, especially in the German reports, not considered. The estimation and interpretation of pressures in auricular fibrillation, as will be shown in another place, present problems much more intricate than those in the sinus rhythm, and the results of investigations indicate a different response to digitalis.

In the group of patients under consideration, those with sinus rhythm, without edema, and with a moderate or low systolic pressure, we have seen no substantial alteration in the level of either the systolic or the diastolic curve. Our conclusions are like those of other more recent observers. The few opportunities we have taken to verify the observations made by Marvin have failed in establishing results similar to his. In the cases we have studied, then, no alteration in pressure was found. In so far as the older fears are concerned, it is apparent that digitalis must be considered a safe drug to use.

Vascular effects, however, aside from changes in pressure, may be produced, and the untoward or beneficial nature of these requires consideration. A specific vascular action, not dependent on heart action or on pressure effects, has been advanced and held by the school of Gottleib. Gottleib, while admitting a cardiac action of digitalis nevertheless maintains that the peripheral action of the drug is the one by virtue of which its prime effects are produced. More recently clinicians, employing optical methods of registration devised by Frank, have again taken up these aspects of digitalis action, and reports are now being published of alterations in reaction of the blood vessels, notably in the tension of the vessel walls, when they are under the action of the drug. It is the results of these investigations, far from satisfying as yet, which demand further study in order to establish the assurance that the vessels of the body, and more especially of the brain and meninges, do not become subject to injury as the result of giving digitalis.

The last phase of the action of digitalis which I wish to discuss

is the relation of the drug to the excretion of urine. The fox-glove obtained its initial reputation because diuresis was so enormously increased when it was administered. For almost a hundred years it was supposed that this was the one and most striking of its effects; but now the situation in regard to this phase of the problem is less simple. The questions now being discussed relate first to when digitalis does, and when it does not, increase the amount of urine, and, second, when it does, by virtue of what mechanism this result is brought about. The first problem, whether or not the amount of urine is increased, is a clinical problem and can be definitely solved. In the patients in the group under consideration, it may be stated definitely that no increase in diuresis takes place. It is, of course, remembered that these patients have no edema. The problem as it relates to patients having edema is naturally different, and involves other factors; but in the simplest form of case, no increase in the amount of urine takes place. So far, digitalis cannot be considered a specific diuretic. In experiments the situation is different, because there, although obviously edema is likewise absent, it is almost universally maintained that increased urinary flow takes place. But the clinical and the experimental conditions are not quite parallel, because in the latter, doses which have usually been employed are much larger than normal, that is to say, from three to fifty and more times the lethal dose for man; and because the method is an acute method, for the drug is given intravenously, or under the skin, and the effect is observed under the conditions of surgical operation. But, although the clinical and experimental conditions present results so divergent, it may be interesting to enumerate the theories which are given in explanation of the occurrences observed in experiments, the more in that they discuss factors which must be considered in the diuresis which occurs when digitalis is given in edema. As may have been supposed, three possible explanations have been urged, first that the experimental diuresis depends on a local vascular action in the renal vessels; second, that it depends on the general elevation in vascular blood pressure, and third, that it depends on the increased force of the heart's action. It will be noticed that none of these explanations refers to an action on renal epithelium. But this phase of the discussion of digitalis action scarcely bears on the problem now being considered, and may consequently be dismissed.

We return to considering the effect of digitalis in patients of our group. Diuresis, as has been said, does not occur in any stage of the treatment. When the cardiac effects are clearly discerned, as in the auriculoventricular interval and in the state of the *T* wave, no alteration in output is found. Intake is, of course, maintained level. But later, when dissociation occurs, and gastro-intestinal symptoms have taken place, the output actually diminishes. On account of loss of appetite, and, in some cases, of nausea and vomiting, the fluid intake naturally falls and accounts satisfactorily for urinary decrease. In view of these considerations, the conclusion is justified that a specific effect on urinary output does not occur as the result of giving digitalis to the class of patients under discussion. This, in itself, is not a significant contribution to an understanding of the pharmacology of the drug, but in another place it will be shown that it has an important bearing on the drug's action in edema.

No consideration of the action of digitalis on the various functions of the heart, on rate, on conduction, on contraction, and on circulation, is complete without a reference to the nature of this action. For many years a war of words, the two most prominent of which were "myogenic" and "neurogenic," was fought. This fight was waged on the question of the conduction of impulses in the heart. The battle became a drawn battle; no decision could be reached. Anatomic studies showed that both nerve and muscle elements were inextricably associated in the heart in general, and in the special muscular systems as well. The effort to distinguish the functions of one tissue from those of another became impossible, and the struggle was abandoned.

The same words, "neurogenic" and "myogenic," have in a similar way formed centers around which another battle has been fought, the battle to elucidate the mechanism of the action of digitalis. Fortunately, these battles have been less violent, and the lesser violence was probably due to the fact that it was always more than half suspected that neither one side nor the other was altogether wrong, and, in fact, that both sides were partially right. Our studies, it seems to us, indicate that this is so. So far as atropin may be taken to be a drug acting especially on the ends of the vagi, a release of the heart from a phase of digitalis action by its use indicates a nerve action of

digitalis (Fig. 1 B). So far as digitalis has other effects, which atropin does not release, it may be inferred that it has an action directed more especially to muscle. Both effects can be observed at the same time, as we have seen, and by virtue of them another struggle, one may hope, has been terminated.

Our studies have, then, led us to draw a number of definite conclusions in regard to the way digitalis acts, and to find new criteria in the *T* wave and in the effect on conduction for detecting when the drug is acting. It seems important to emphasize the fact that it is essential to distinguish differences which patients suffering from heart disease present, and to study them in groups, with these differences in mind. Rhythm certainly offers a prime basis. The effect of digitalis on rate and on a number of other capacities varies with the nature of the disturbed function. In the use of the drug in pneumonia we have found it to have an action essentially similar to that found in the nonfebrile. In the absence of edema, we have not observed a diuretic effect. Evidence has been found which indicates persistence of action, longer than can be accounted for if the drug acts only by virtue of its concentration. We have shown that digitalis has a twofold action on the heart, nervous and muscular. We believe that clearness of indications will result only if the exact mechanism of the action of the drug is ascertained; for only when these are clearly met, can rational treatment be instituted.

## A FURTHER STUDY OF THE BACTERICIDAL ACTION OF ETHYLHYDROCUPREIN ON PNEUMOCOCCI.

By HENRY F. MOORE, M.B., B.Ch., B.A.O.

(From the Hospital of The Rockefeller Institute for Medical Research.)

(Received for publication, September 1, 1915.)

In a previous communication<sup>1</sup> we stated that ethylhydrocuprein inhibits the growth of, and kills, pneumococci *in vitro* in very considerable dilutions of the drug, and that it exerts a considerable protective action in experimental pneumococcal infections in mice. The present study was undertaken with the object of gaining some information as to the rate of absorption of the drug into the circulation in the animal body, as to how long the resulting bactericidal effect, if any, of the serum on pneumococci lasted, and as to the mode of action of the drug on these microorganisms.

Ethylhydrocuprein, a derivative of hydroquinine, was introduced by Morgenroth<sup>2</sup> in 1911 in the treatment of experimental pneumococcal infection in mice. It has since been subjected to study by many observers, who, generally speaking, agree that it has a bactericidal action on pneumococci, *in vitro* and *in vivo*. Wright<sup>3</sup> showed that the blood serum of mice previously treated with the drug killed pneumococci in the test-tube.

In the present study we considered it advisable to make use of rabbits as our experimental animals, and, having determined the tolerated and toxic doses of the hydrochloride of the drug (optochin hydrochloride) and of the free base (optochin base) for these animals, we proceeded to study the action of each of these preparations when given by different routes as set forth below. The hydrochloride was given to the rabbits subcutaneously, dissolved in 5 cc. of

<sup>1</sup> Moore, H. F., *Jour. Exper. Med.*, 1915, xxii, 269.

<sup>2</sup> Morgenroth, J., and Levy, R., *Berl. klin. Wchnschr.*, 1911, xlviii, 1560, 1979.

<sup>3</sup> Wright, A. E., Morgan, W. P., Colebrook, L., and Dodgson, R. W., *Lancet*, 1912, ii, 1633, 1701.

distilled water; intravenously, dissolved in 10 cc. of physiological salt solution; and by mouth, dissolved in 25 cc. of distilled water. The free base was given subcutaneously and intramuscularly dissolved in from 5 to 6 cc. of sterile olive oil.

#### EXPERIMENTAL.

Our experiments show that normal rabbits of approximately 2,000 grams tolerate a single dose of 0.1 gram of the hydrochloride given subcutaneously and 0.125 gram of the base in oil given in the same manner, per kilo of body weight. It seems, however, that the tolerance of normal rabbits of greater weight—3,000 grams and upwards—is less than this. The tolerance of normal rabbits of about 2,000 grams' weight for a single dose of the base in oil when this solution is given intramuscularly (into the erector spinæ mass) is still lower—0.075 gram per kilo of body weight. Finally, the animals are able to bear, without showing signs of toxicity, only a small dose of the hydrochloride dissolved in normal salt solution given slowly intravenously; namely, a dose lying between 0.02 and 0.05 of a gram per kilo of body weight. In this case the drug must be given well diluted and very slowly; for, if given in any considerable concentration, a reaction between it and the blood plasma takes place with a resulting heavy precipitate which causes speedy death.

We have observed that the tolerance of rabbits previously infected with pneumococci seems to be somewhat less than that of normal animals.

The drug in a toxic dosage gives rise to certain characteristic symptoms in rabbits, the variety and intensity of which depend in part on the dose, in part on the route by which it is given, and in part on the location of the injection. In order of severity, in the case of subcutaneous or intramuscular injection, the symptoms are as follows: quietness of the animal and disinclination to eat; halting movement of the legs nearest the side of injection; spastic and incoordinated movements of the same; and complete paralysis of the extremities nearest the injection site. Finally, the paralysis may spread to the other limbs and the animal may lie on the floor collapsed, and die. The respirations are at first hurried and, with a larger dose, later diminish in rate, and symptoms of dyspnea may



appear. When a toxic dose is given intravenously, the animal shows convulsive movements, more or less severe according to the size of the dose, and may finally die with convulsions and exophthalmos. The bactericidal action of the drug described below is seen in the serum of animals showing severe toxic signs as well as in animals which received a dose well below the toxic limit.

*Technique.*—Normal rabbits weighing about 2,000 grams were used. Each rabbit was bled from the marginal ear vein, the ear having previously been thoroughly cleansed with bichloride of mercury and alcohol and wiped dry with a sterile sponge. The drug was then administered in the manner and amount mentioned in each protocol below. The animals were bled either as before from the marginal ear vein, or directly from the heart—which latter may be done with safety many times—at stated intervals after the administration of the drug. The blood in each case received in sterile centrifuge tubes was, after the final bleeding, placed in the ice chest until the following morning, when the clot was loosened and centrifugalized. 3 cc. of serum from each centrifuge tube were pipetted off the clot into a test-tube, a separate pipette being used in each case, and inactivated for one-half hour at 56° C. in a water bath. (In a few special cases the active serum was used with the object of comparing the bactericidal power of such serum with the same serum inactivated, as described above.) The 3 cc. of serum in each test-tube were inoculated with 0.5 cc. of a dilution of an 18 hour broth culture of a pneumococcus of from 1 in 100,000 to 1 in 1,000,000. Stock strains of pneumococci of Groups I and II were used, generally the latter. The pneumococci having been thoroughly mixed with the serum, 0.5 cc. of the mixture was plated in about 10 cc. of 1 per cent glucose agar, which had been previously melted and cooled to 40° C. By this means the drug, even if present in the serum in sufficient concentration to prevent the growth, or cause death, of the pneumococci, was so diluted in the plate as to leave the pneumococci free to grow unhindered. The Petri dishes used were 10 cm. in diameter. The plates were incubated for from 20 to 24 hours, at 37° C., and at the end of this period the number of colonies in each was counted. The tubes containing the inoculated serum were also incubated at 37° C. for definite periods, as stated in the

tables, at the end of each of which periods 0.5 cc. of the contents of each tube was plated as before, and these plates were incubated in the same way. In this way we were enabled to gain information on the bactericidal action on pneumococci of the serum of animals treated with ethylhydrocuprein in relation to the points already stated.

*Explanation of the Protocols.*—The numerals in the protocols represent the number of colonies resulting from plating 0.5 cc. of the inoculated serum from the corresponding test-tube either immediately after the tubes were inoculated, or after a definite period of incubation; the figures in the vertical columns correspond to the intervals between the administration of the drug and the various bleedings of the animal; the figures in each horizontal row represent the number of colonies per 0.5 cc. of serum from each particular bleeding after a definite period of incubation.

*Experiments Illustrating the Effects on Pneumococci of the Serum of Animals Treated with Ethylhydrocuprein Hydrochloride (Optochin Hydrochloride) Dissolved in Water, Given Subcutaneously (under the Skin of the Back).*

*Experiment 1.*—(Table I.) Rabbit 98 E; weight 1,600 gm. Received 0.1 gm. of ethylhydrocuprein hydrochloride per kilo of body weight, in 5 cc. of distilled water, subcutaneously. No toxic symptoms. *Pneumococcus*: stock strain of Group II.

TABLE I.

Serum obtained.	No. of colonies per 0.5 cc. when plated immediately after inoculation.	No. of colonies when plated after 7 hrs.' incubation.	No. of colonies when plated after 22½ hrs.' incubation.
Before giving drug. . . . .	119	Almost infinity	Infinity.
1½ hrs. after. . . . .	105	36	0
4 " " . . . . .	126	Almost infinity	Infinity.
5 " " . . . . .	116	" "	"

*Experiment 2.*—(Table II.) Rabbit 115 D; weight 1,900 gm. Received 0.1 gm. of ethylhydrocuprein hydrochloride per kilo of body weight, in 5 cc. of distilled water, subcutaneously. No toxic symptoms. *Pneumococcus*: stock strain of Group II.

TABLE II.

Serum obtained.	No. of colonies per 0.5 cc. when plated immediately after inoculation.	No. of colonies when plated after 7½ hrs.' incubation.	No. of colonies when plated after 30 hrs.' incubation.
Before giving drug. . . . .	410	Infinity	Infinity.
1½ hrs. after. . . . .	445	29	3
2½ " " . . . . .	492	176	Infinity.

*Experiment 3.*—(Table III.) Rabbit 95 E; weight 2,200 gm. Received 0.1 gm. of ethylhydrocuprein hydrochloride per kilo of body weight, in 5 cc. of distilled water, subcutaneously. No toxic symptoms. Pneumococcus: stock strain of Group II.

TABLE III.

Serum obtained.	No. of colonies per 0.5 cc. when plated immediately after inoculation.	No. of colonies when plated after 6½ hrs. incubation.	No. of colonies when plated after 21 hrs. incubation.
Before giving drug . . . . .	329	Almost infinity	Infinity.
1 hr. after . . . . .	268	46	18
2 " " . . . . .	273	135	146
4½ " " . . . . .	316	Several thousand	Infinity.

*Experiment 4.*—(Table IV.) Rabbit 141 D; weight 2,130 gm. Received 0.1 gm. of ethylhydrocuprein hydrochloride per kilo of body weight, in 5 cc. of distilled water, subcutaneously. No toxic symptoms. Pneumococcus: stock strain of Group I rendered highly virulent for rabbits by passage.

TABLE IV.

Serum obtained.	No. of colonies per 0.5 cc. when plated immediately after inoculation.	No. of colonies when plated after 6 hrs. incubation.	No. of colonies when plated after 22 hrs. incubation.
Before giving drug . . . . .	281	Almost infinity	Infinity.
1½ hrs. after . . . . .	351	62	0
2½ " " . . . . .	285	134	Several thousand.
3½ " " . . . . .	282	188	Infinity.
4½ " " . . . . .	297	506	"
5½ " " . . . . .	306	Almost infinity	—

*Experiment 5.*—(Table V.) Rabbit 2; weight 2,300 gm. Received 0.2 gm. of ethylhydrocuprein hydrochloride per kilo of body weight, in 5 cc. of distilled water, subcutaneously. Severe toxic appearances; died immediately after last bleeding. Pneumococcus: stock strain of Group II.

TABLE V.

Serum obtained.	No. of colonies per 0.5 cc. when plated immediately after inoculation.	No. of colonies after 2½ hrs. incubation.	No. of colonies after 4 hrs. incubation.	No. of colonies after 6½ hrs. incubation.	No. of colonies after 10½ hrs. incubation.
Before giving drug . . . . .	456	478	Almost infinity	Infinity	—
2½ hrs. after . . . . .	460	382	245	35	1
7½ " " . . . . .	478	456	390	379	127

*Experiment 6.*—(Table VI.) Rabbit 3; weight 1,700 gm. Received 0.15 gm. of ethylhydrocuprein hydrochloride per kilo of body weight, in 5 cc. of distilled water, subcutaneously. Severe toxic symptoms; died immediately after last bleeding. Pneumococcus: stock strain of Group II.

TABLE VI.

Serum obtained.	No. of colonies per 0.5 cc. when plated immediately after inoculation.	No. of colonies after 2 hrs. incubation.	No. of colonies after 5 hrs. incubation.	No. of colonies after 11 hrs. incubation.	No. of colonies after 24 hrs. incubation.
Before giving drug . . . . .	187	188	Almost infinity	Infinity	Infinity.
1½ hrs. after . . . . .	194	127	64	0	0
2½ " " . . . . .	187	175	123	56	46
4½ " " . . . . .	192	181	212	Several thousand	Infinity.

*Comment on Experiments 1 to 6, Inclusive.*—A study of the figures shown in the protocols of these experiments reveals certain facts. The serum of animals given a suitable dose (*e. g.*, 0.1 gram per kilo of body weight) of ethylhydrocuprein hydrochloride subcutaneously has a strongly bactericidal action *in vitro* on the pneumococci, these microorganisms growing freely in normal rabbit serum. This property is possessed by serum obtained as early as one hour after the administration of the drug—in fact, it is at its maximum about this time, or a little later; having attained a maximum potency the bacteriolytic effect gradually falls off, and, in the case of a dose of 0.1 gram per kilo of body weight (a dose well tolerated), it passes into an inhibitory effect on the growth of the pneumococci within from 2 to 2½ hours after the animal received the drug; the inhibitory effect seems to restrain the free growth of the microorganisms for some hours, after which this action seems to be overcome; this inhibitory effect on growth, in its turn, disappears about 5 hours after the drug has been given. Further, the killing off of the pneumococci in the serum of animals treated with the drug is a gradual process, lasting several hours, according to the dosage of the drug and the amount of inoculation, etc. The same effects are to be seen in the serum of animals which have received a toxic dose of the drug, except that the duration of these actions is longer, and, perhaps, more powerful.

*Experiments Illustrating the Effects on Pneumococci of the Serum of Animals Treated with the Free Base Ethylhydrocuprein (Optochin Base) Dissolved in Sterile Olive Oil, Given Subcutaneously (under the Skin of the Back).*

*Experiment 7.*—(Table VII.) Rabbit 142 D; weight 2,200 gm. Received 0.075 gm. of ethylhydrocuprein base per kilo of body weight, in 6 cc. of olive oil, subcutaneously. No toxic symptoms. Pneumococcus: stock strain of Group I rendered highly virulent for rabbits by passage.

TABLE VII.

Serum obtained.	No. of colonies per 0.5 cc. when plated immediately after inoculation.	No. of colonies after 4 hrs.' incubation.	No. of colonies after 22 hrs.' incubation.
Before giving drug.....	301	Almost infinity	Infinity.
1 hrs. after.....	305	116	0
" ".....	290	227	Almost infinity.
" ".....	297	189	" "
" ".....	351	408	Infinity.
" ".....	358	Several thousand	—

*Experiment 8.*—(Table VIII.) Rabbit 91 E; weight 2,200 gm. Received 0.1 gm. of ethylhydrocuprein base per kilo of body weight, in 6 cc. of olive oil, subcutaneously. No toxic symptoms. Pneumococcus: stock strain of Group II.

TABLE VIII.

Serum obtained.	No. of colonies per 0.5 cc. when plated immediately after inoculation.		No. of colonies after 4 hrs.' incubation.		No. of colonies after 22 hrs.' incubation.	
	Serum inactivated.	Serum not inactivated.	Serum inactivated.	Serum not inactivated.	Serum inactivated.	Serum not inactivated.
Before giving drug.....	321	—	Almost infinity	—	Infinity	—
1 hr. after.....	342	292	94	74	1	0
2½ " ".....	382	350	158	162	98	37
4½ " ".....	305	323	207	257	Several thousand	Almost infinity.

*Experiment 9.*—(Table IX.) Rabbit 105 D; weight 1,750 gm. Received 0.1 gm. of ethylhydrocuprein base per kilo of body weight, in 6 cc. of olive oil, subcutaneously. No toxic symptoms. Pneumococcus: stock strain of Group I.

TABLE IX.

Serum obtained.	No. of colonies per 0.5 cc. when plated immediately after inoculation.	No. of colonies after 3 hrs.' incubation.	No. of colonies after 7½ hrs.' incubation.	No. of colonies after 20 hrs.' incubation.
Before giving drug...	573	Several thousand	Infinity	—
2 hrs. after.....	349	200	41	0
3 " " .....	402	224	76	3
4½ " " .....	400	270	123	17
5½ " " .....	410	238	138	8

*Experiment 10.*—(Table X.) Rabbit 106 D; weight 1,700 gm. Received 0.1 gm. of ethylhydrocuprein base per kilo of body weight, in 6 cc. of olive oil, subcutaneously. No toxic symptoms. Pneumococcus: stock strain of Group I.

TABLE X.

Serum obtained.	No. of colonies per 0.5 cc. when plated immediately after inoculation.	No. of colonies after 3 hrs.' incubation.	No. of colonies after 7½ hrs.' incubation.	No. of colonies after 20 hrs.' incubation.
Before giving drug...	397	Several thousand	Infinity	—
2 hrs. after.....	330	197	59	0
3 " " .....	338	229	125	14
4½ " " .....	358	298	190	552*
5½ " " .....	374	460	—	—

\* Macroscopically there appeared to be no growth in the corresponding tube.

*Experiment 11.*—(Table XI.) Rabbit 104 D; weight 1,650 gm. Received 0.125 gm. of ethylhydrocuprein base per kilo of body weight, in 6 cc. of olive oil, subcutaneously. Animal showed a tendency to lie quiet for some hours after the injection. Pneumococcus: stock strain of Group I.

TABLE XI.

Serum obtained.	No. of colonies per 0.5 cc. when plated immediately after inoculation.	No. of colonies after 3 hrs.' incubation.	No. of colonies after 7½ hrs.' incubation.	No. of colonies after 20 hrs.' incubation.
Before giving drug...	594	Several thousand	Infinity	Infinity.
2 hrs. after.....	520	149	51	0
3½ " " .....	419	159	107	0
4½ " " .....	368	256	1,200	Infinity.

*Experiment 12.*—(Table XII.) Rabbit 101 D; weight 1,720 gm. Received 0.15 gm. of ethylhydrocuprein base per kilo of body weight, in 6 cc. of olive oil, subcutaneously. Animal apparently well up to 6 hours after injection; died during the last bleeding. Pneumococcus: stock strain of Group II.

TABLE XII.

Serum obtained.	No. of colonies per 0.5 cc. when plated immediately after inoculation.	No. of colonies after 5½ hrs.' incubation.	No. of colonies after 8½ hrs.' incubation.	No. of colonies after 25 hrs.' incubation.
Before giving drug...	976	Almost infinity	Infinity	Infinity.
2 hrs. after.....	1,100	180	57	0
3½ " ".....	1,328	386	292	0
5 " ".....	1,152	403	283	Almost infinity.
6 " ".....	1,284	Several thousand	Infinity	Infinity.

*Experiment 13.*—(Table XIII.) Rabbit 102 D; weight 1,770 gm. Received 0.15 gm. of ethylhydrocuprein base per kilo of body weight, in 6 cc. of olive oil, subcutaneously. No toxic symptoms. Pneumococcus: stock strain of Group II.

TABLE XIII.

Serum obtained.	No. of colonies per 0.5 cc. when plated immediately after inoculation.	No. of colonies after 5½ hrs.' incubation.	No. of colonies after 8½ hrs.' incubation.	No. of colonies after 25 hrs.' incubation.
Before giving drug...	1,176	Infinity	Infinity	Infinity.
2 hrs. after.....	1,232	452	224	10
3½ " ".....	958	339	169	Infinity.
5 " ".....	1,152	500	441	Almost infinity.

*Experiment 14.*—(Table XIV.) Rabbit 121 F; weight 2,050 gm. Received 0.15 gm. of ethylhydrocuprein base per kilo of body weight, in 6 cc. of olive oil, subcutaneously. No toxic symptoms. Pneumococcus: stock strain of Group II.

TABLE XIV.

Serum obtained.	No. of colonies per 0.5 cc. when plated immediately after inoculation.	No. of colonies after 5½ hrs.' incubation.	No. of colonies after 23 hrs.' incubation.
Before giving drug.....	68	Almost infinity	Infinity.
2 hrs. after.....	58	0	0
3 " ".....	64	0	0
4 " ".....	59	7	0
5 " ".....	43	18	0

*Comment on Experiments 7 to 14, Inclusive.*—These protocols, like those of Experiments 1 to 6, show that the serum of animals given subcutaneously a suitable dose of ethylhydrocuprein base (optochin base) dissolved in olive oil has a strongly bactericidal action *in vitro* on the pneumococci, that this property is possessed by serum obtained as early as 1 hour after the administration of the drug, and

that having attained a maximum potency the bacteriolytic effect gradually falls off. In the case of a dose of 0.1 gram per kilo of body weight (a dose well tolerated), the bactericidal action passes into an inhibitory effect on the growth of the pneumococci in about four hours after the animal received the drug; here again, the inhibitory effect seems to restrain the free growth of the microorganism for some hours, after which this action seems to be overcome. The inhibitory effect, in its turn, ultimately disappears. As before, the protocols show that the killing off of the pneumococci in the serum of the treated animals is a gradual process. The effects are more lasting than when the hydrochloride in water is given by the same route.

*Experiments Showing the Effects on Pneumococci of the Serum of Animals Treated with Ethylhydrocuprein Hydrochloride Dissolved in 10 Cc. of Normal Saline Solution and Given Slowly Intravenously.*

*Experiment 15.*—(Table XV.) Rabbit 9; weight 2,000 gm. Received 0.02 gm. of ethylhydrocuprein hydrochloride per kilo of body weight, in 10 cc. of normal saline, slowly, intravenously.

Slight convulsions toward end of injection; immediately after injection the animal lay stretched out on floor and apparently could not rise. Complete recovery in a few minutes. *Pneumococcus*: stock strain of Group II.

TABLE XV.

Serum obtained,	No. of colonies per 0.5 cc. when plated immediately after inoculation.	No. of colonies after 7 hrs.' incubation.	No. of colonies after 28 hrs.' incubation.
Before giving drug . . . . .	405	Infinity	Infinity.
5 min. after . . . . .	426	533	About 2,000.
1½ hrs. " . . . . .	360	Infinity.	Infinity.

*Experiment 16.*—(Table XVI.) Rabbit 10; weight 2,700 gm. Received 0.078 gm. of ethylhydrocuprein hydrochloride per kilo of body weight, in 10 cc. of normal saline, slowly, intravenously.

Convulsions and exophthalmos toward the end of the injection; immediately after injection the animal lay on floor and seemed collapsed; twitching of legs. Recovered in a few minutes. *Pneumococcus*: stock strain of Group II.



TABLE XVI.

Serum obtained	No. of colonies per 0.5 cc. when plated immediately after inoculation.	No. of colonies after 3½ hrs. incubation.	No. of colonies after 7 hrs. incubation.	No. of colonies after 20 hrs. incubation.
Before giving drug...	304	249	Infinity	Infinity.
5 min. after.....	329	235	87	10
1½ hrs. " .....	340	315	Infinity	—
4½ " " .....	350	292	"	—

The serum of rabbits which had been given intravenously doses of the hydrochloride smaller than those given to Rabbits 9 and 10 showed no bactericidal or inhibitory effect on the pneumococci. An animal given 0.05 gm. per kilo of body weight intravenously died immediately after the injection with convulsions.

*Comment on Experiments 15 and 16.*—The serum of rabbits given a non-fatal dose of hydrochloride of the drug intravenously does not show such a strong or prolonged effect on pneumococci as when the drug is given subcutaneously. When given intravenously, toxic signs are more easily obtained than when other routes are used.

*Experiments Showing the Effects on Pneumococci of the Serum of Animals Treated with Ethylhydrocuprein Base (Optochin Base), Dissolved in Olive Oil, and Given Intramuscularly.*

*Experiment 17.*—(Table XVII.) Rabbit 120 D; weight 1,620 gm. Received 0.075 gm. of ethylhydrocuprein base per kilo of body weight, in 6 cc. of olive oil, intramuscularly. No toxic symptoms. Pneumococcus: stock strain of Group II.

TABLE XVII.

Serum obtained.	No. of colonies per 0.5 cc. when plated immediately after inoculation.	No. of colonies after 10 hrs. incubation.	No. of colonies after 22 hrs. incubation.
Before giving drug.....	61	Infinity	Infinity.
1½ hrs. after.....	60	142	"
3½ " " .....	71	Almost infinity	"
4½ " " .....	77	Infinity	"
4½ " " .....	73	"	"

*Experiment 18.*—(Table XVIII.) Rabbit 121 D; weight 1,850 gm. Received 0.075 gm. of ethylhydrocuprein base per kilo of body weight, in 6 cc. of olive oil, intramuscularly. No toxic symptoms. Pneumococcus: stock strain of Group II.

TABLE XVIII.

Serum obtained.	No. of colonies per 0.5 cc. when plated immediately after inoculation.	No. of colonies after 10 hrs.' incubation.	No. of colonies after 22 hrs.' incubation.
Before giving drug . . . . .	74	Infinity	Infinity.
1½ hrs. after . . . . .	64	15	2
3½ " " . . . . .	91	Several thousand	Infinity.
4½ " " . . . . .	91	Almost infinity	"
5 " " . . . . .	89	Infinity	"

*Experiment 19.*—(Table XIX.) Rabbit 169 D; weight 2,000 gm. Received 0.1 gm. of ethylhydrocuprein base per kilo of body weight, in 6 cc. of olive oil, intramuscularly. Distinct toxic symptoms within 2 hours after administration of drug. Recovered next day. *Pneumococcus*: stock strain of Group II.

TABLE XIX.

Serum obtained.	No. of colonies per 0.5 cc. when plated immediately after inoculation.	No. of colonies after 4½ hrs.' incubation.	No. of colonies after 21 hrs.' incubation.
Before giving drug . . . . .	1,012	Almost infinity	Infinity.
1½ hrs. after . . . . .	984	124	1
2½ " " . . . . .	952	413	Infinity.
4½ " " . . . . .	1,010	Several thousand	"

*Comment on Experiments 17 to 19, Inclusive.*—These experiments, illustrating the results of giving the base of the drug dissolved in oil intramuscularly, show that the drug is more toxic when given by this route than when given subcutaneously in the same vehicle and that the bactericidal effect on the pneumococci is not so prolonged, and probably not so intense, as when the subcutaneous route is used.

In addition to the experiments mentioned above, we have studied the bactericidal action of the serum of rabbits into the stomachs of which the hydrochloride of the drug, dissolved in 25 cc. of distilled water, was introduced by means of a stomach tube; the tolerance is greatest by this route, but the bactericidal effects are slight or absent; even by giving doses as large as 0.3 to 0.4 gram per kilo of body weight to rabbits of 2,000 grams' weight, and otherwise using the same technique as described above, we have not been able to demonstrate any bactericidal action of the serum of these animals on pneumococci within a period of from 2½ to 6 hours after administration of the drug, and only a slight inhibitory effect on their growth.

In order to gain some idea of the bactericidal action on pneumococci of the serum of man treated with the drug the experiments immediately to be described were carried out. The technique was the same as that described above.

*Experiment 20.*—(Table XX.) Normal man, M.; weight 50.8 kilos. Received, *per os*, in capsules, 0.5 gm. of ethylhydrocuprein hydrochloride. No toxic signs or symptoms. Pneumococcus: stock strain of Group II.

TABLE XX.

Serum obtained.	No. of colonies per 0.5 cc. when plated immediately after inoculation.	No. of colonies after 2½ hrs. incubation.	No. of colonies after 8 hrs. incubation.	No. of colonies after 12 hrs. incubation.	No. of colonies after 21 hrs. incubation.
Before giving drug . .	395	3,000 (approximately)	Infinity	Infinity	—
1 hr. after . . . . .	458	382	391	1,500	Several thousand.
2 " " . . . . .	560	311	337	3,000 (approximately)	Several thousand.
5 " " . . . . .	374	400	Several thousand	Almost infinity	Almost infinity.

*Experiment 21.*—(Table XXI.) Normal man, A; weight 68.96 kilos. Received *per os*, in capsules, 0.5 gm. of ethylhydrocuprein hydrochloride. No toxic signs or symptoms. Pneumococcus: stock strain of Group II.

TABLE XXI.

Serum obtained.	No. of colonies per 0.5 cc. when plated immediately after inoculation.	No. of colonies after 4½ hrs. incubation.	No. of colonies after 9½ hrs. incubation.	No. of colonies after 21 hrs. incubation.
Before giving drug . .	517	Almost infinity	Infinity	Infinity.
1½ hrs. after . . . . .	560	540	Almost infinity	"
2½ " " . . . . .	521	230	225	Almost infinity.
5½ " " . . . . .	518	483	Several thousand	Infinity.

*Experiment 22.*—(Table XXII.) Man, B.; weight 52 kilos. Received 0.5 gm. of ethylhydrocuprein hydrochloride dissolved in 5.0 cc. of sterile redistilled water, subcutaneously, in left flank. Next day complained of pains in legs, had a slight rise of temperature, and the area around the injection site was hyperemic and showed a slight superficial edema. Pneumococcus: stock strain of Group II.

TABLE XXII.

Serum obtained,	No. of colonies per 0.5 cc. when plated immediately after inoculation,		No. of colonies after 3 hrs.' incubation.		No. of colonies after 8½ hrs.' incubation.		No. of colonies after 21 hrs.' incubation.	
	Serum inactivated.	Serum not inactivated.	Serum inactivated.	Serum not inactivated.	Serum inactivated.	Serum not inactivated.	Serum inactivated.	Serum not inactivated.
Before giving drug....	76	112	1,300	2,000	Almost infinity	Infinity	Infinity	Infinity.
1 hr. after.....	68	54	25	40	34	19	Almost infinity	Infinity.
2 " ".....	65	52	29	27	22	140	Infinity	Infinity.
3 " ".....	61	68	38	37	153	467	"	Infinity.
4½ " ".....	56	59	24	31	713	431	"	Infinity.

*Experiment 23.*—(Table XXIII.) Woman, S.; weight 51 kilos. Received 0.5 gm. of ethylhydrocuprein hydrochloride dissolved in 5 cc. of sterile redistilled water, subcutaneously, in abdominal wall. Next day the patient complained of general malaise and headache, had a slight rise of temperature, and had a painful hyperemic area of infiltration and edema about 3 inches in diameter around the injection site which disappeared in a few days. Pneumococcus: stock strain of Group II.

TABLE XXIII.

Serum obtained,	No. of colonies per 0.5 cc. when plated immediately after inoculation.	No. of colonies after 3 hrs.' incubation.	No. of colonies after 8½ hrs.' incubation.	No. of colonies after 21 hrs.' incubation.
Before giving drug.....	60	51	Almost infinity	Infinity.
1 hr. after.....	56	35	1,320	"
2½ " ".....	74	34	439	"

*Comment on Experiments 20 to 23, Inclusive.*—The figures show that the serum of a man to whom had been given a single dose of 0.5 gram of the hydrochloride of the drug either by the mouth or subcutaneously, has a decided inhibitory effect on the growth of the pneumococcus lasting for several hours, after which this action seems to be overcome, and has a bactericidal action which is much less strong than that shown in the protocols of the animal experiments described above. Similar effects are seen whether the drug be administered subcutaneously or by mouth. The employment of the

former method, however, subjects the patient to considerable discomfort.

The serum of a normal man to whom 1.8 grams of the hydrochloride of the drug were given by the mouth in 24 hours, that is, in four doses of 0.45 gram each given at regular intervals, showed a cumulative effect; namely, the bactericidal action was much stronger  $2\frac{3}{4}$  hours after the last, than at a similar period after the first, dose.

#### DISCUSSION OF RESULTS.

In serum, the pneumococci show a tendency to form short chains composed of from two to four diploid forms. The figures in the foregoing protocols consequently must be regarded, not as giving the total number of diplococci per 0.5 cc. of the inoculated serum, but as an index to that number. The figures, in other words, are relative, not absolute, but the conclusions drawn with regard to the bactericidal action of ethylhydrocuprein are perfectly valid, for the growth in normal serum serves as a control. Moreover, we have cumulative evidence of this bactericidal action of the drug in the fact that the progressive diminution in the number of pneumococci is seen in a considerable number of different experiments. Again, this progressive diminution frequently goes to the point of complete disappearance of living pneumococci in a few hours.

The protocols, therefore, set forth above show that the serum of rabbits treated with ethylhydrocuprein exerts a bactericidal action on the pneumococci in the test-tube, and later inhibits their growth. The intensity and the time of appearance and disappearance of these actions depend on the dosage, upon the route by which the drug is given, and on the method of administration. With the dosage used in the experiments above described, these effects last longest when the base is given in oil subcutaneously; they are not quite so lasting when the hydrochloride is given in water, subcutaneously; and are still less lasting when the base is given in oil, intramuscularly. To obtain bactericidal effects in rabbits by giving the hydrochloride dissolved in physiological saline intravenously, one must give toxic doses dangerous to the life of the animal. The giving of the hydrochloride directly into the stomach of rabbits does not seem to be efficient from the point of view of a resulting bactericidal action of the serum on the pneumococci.

It was thought that a possible explanation of the progressive decrease in the number of colonies in those tubes in which this was demonstrated might be an agglutinative effect of the drug on the pneumococci. We have frequently examined the contents of the tubes microscopically, macroscopically, and by cultural methods for sterility (the latter in the cases in which the plates showed no colonies), and we have never found any evidences of agglutination.

It will be noticed in the protocols of several experiments that, in the case of serum obtained when, apparently, the optochin concentration in the blood is diminishing, the number of colonies in the incubated tubes at first shows a progressive decrease (the number in the control normal serum, obtained before giving the drug, showing, at the same time, a progressive increase); but, that, later, after a few hours' incubation, the pneumococci progressively increase, until they become too numerous to count in the plates. This phenomenon is more apparent in tubes lightly inoculated than in tubes more heavily inoculated with pneumococci. Evidently, if the optochin concentration in the serum falls below a certain point in relation to the number of pneumococci present, the pneumococci which survive the bactericidal action may, after a few hours in contact with the drug in the incubator, acquire the property of overcoming this action of the drug and, therefore, grow freely. It would be interesting to investigate this property, so rapidly acquired, from the point of view of fastness. It is not due to a destruction of the bactericidal action of the serum caused by simple incubation, because serum incubated for 24 hours, and then inoculated and studied as above described, shows the same intensity of bactericidal action on pneumococci as samples of the same serum not previously subjected to incubation before inoculation. This overcoming of the action of the drug seems to be an entirely new, and, relatively, quickly acquired property of the pneumococci themselves.

It does not seem that there is any considerable difference in the bacteriolytic or inhibitory effects of serum obtained after administration of the drug whether such serum be used after having been inactivated for  $\frac{1}{2}$  hour at  $56^{\circ}$  C., or active.

## CONCLUSIONS.

1. The serum of rabbits which have been previously treated with a single dose of ethylhydrocuprein (optochin) exerts a bactericidal action on, and, later, inhibits the growth of pneumococci in the test-tube.

2. These actions are most evident in the serum of rabbits when the base (optochin base) is given in oil subcutaneously; somewhat less when the hydrochloride of the drug is given in water subcutaneously; slight when the base is given in oil intramuscularly; and least evident, or absent, when the hydrochloride in water is introduced directly into the stomach. To get these effects by the intravenous route, toxic doses must be given, and, even with toxic non-fatal doses, the effects do not last long.

3. In the case of the base given in oil subcutaneously to rabbits in a dosage of 0.1 gram per kilo of body weight, the bactericidal action of the serum is at its maximum about one hour after administration, and it passes into an inhibitory effect about four hours after the drug has been given.

4. In man the same inhibitory and bactericidal actions of the serum are present when a single dose of 0.5 gram of the hydrochloride of the drug is given by the mouth or subcutaneously, but the bactericidal action is not so marked as in rabbits.

5. When the optochin concentration in the serum has, apparently, diminished to a certain point in relation to the number of pneumococci present, the pneumococci which have survived the bactericidal action for a few hours acquire the power of growing freely.

## THE ACTION OF THE LETHAL DOSE OF STROPHANTHIN IN NORMAL ANIMALS AND IN ANIMALS INFECTED WITH PNEUMONIA.

By ROSS A. JAMIESON, M.B.

*(From the Hospital of The Rockefeller Institute for Medical Research.)*

PLATES 74 TO 76.

(Received for publication, July 15, 1915.)

The following experiments were undertaken for the purpose of ascertaining whether the action of a digitalis body when administered to animals suffering from pneumonia differed from its action when no infection or fever was present. That there is a difference of opinion regarding the action of the drug when given in acute infections is shown in the practice of clinicians.

For example, Mackenzie (1) doubts its value and says that digitalis "is often used when patients are dying of some grave affection, as pneumonia, as a last resort, probably more for the purpose of doing something than with expectation of great benefit. I have never seen much good follow the administration of digitalis in acute febrile states." Gibson (2) in his article on acute pneumonia also says, regarding the use of digitalis, that in the worst cases of pneumococcal poisoning the heart absolutely refuses to respond. On the other hand, in Krehl's clinic (3) digitalis is used in all cases of pneumonia with the belief that it has a beneficial effect.

On account of this difference of opinion, Gunn (4) recently performed experiments with the view of determining what influence the height of the temperature has on the action of digitalis. In his experiments he perfused excised rabbits' hearts with solutions of strophanthin at different temperatures. The perfusion was carried to the point of arrest of the heart's action. By this procedure Gunn demonstrated that with variations in temperature ranging from 28° to 41° C. strophanthin acts more quickly on the isolated heart as the temperature is raised. The conditions in Gunn's experiments are not analogous to those in pneumonia, since in the presence of this infection toxemia accompanies the increased temperature.

In the experiments now reported the attempt was made to duplicate the usual human conditions as nearly as possible. A digitalis



body was administered to animals suffering from an acute pneumonic infection and the effect compared with that obtained when the drug was given to healthy animals of the same species. A comparison of the results in the two groups we believe should yield information regarding the influence of infection on the action of the drug.

### *Methods.*

The animals employed were cats and dogs. In each species one group was infected and another group was used as a control. The animals were infected by the method of intrabronchial insufflation described by Lamar and Meltzer (5). The organism used was an eighteen to twenty-four hour old broth culture of virulent pneumococci<sup>1</sup> belonging either to Type I or II (6), and was obtained from the laboratory of the Hospital. Of these cultures the cats received from 10 to 20 cc., and the dogs from 20 to 30 cc. The temperature of the animals was taken daily for several days before infection and also during the course of the disease.

The digitalis body employed was Thoms crystalline g-strophanthin (Merck). An alcoholic stock solution of the drug was made and from this an aqueous solution was prepared for immediate use. In the experiments on cats the aqueous solution was of such a strength that 10 cc. of solution contained 0.1 mg. of strophanthin. For the dogs a solution double this strength was employed. In all experiments the strophanthin was given intravenously. The animal was lightly anesthetized with ether, the femoral vein exposed, and a small glass cannula was inserted. This was connected with a burette containing the solution. The anesthetic was not continued beyond the time necessary for the insertion of the cannula. In injecting the strophanthin, an endeavor was made to maintain a uniform rate of flow. A lethal dose was given to each animal in approximately one hour, unless the animal died before the time required for administering the lethal dose had elapsed. The amount of strophanthin injected differed slightly in cats and dogs. In a large series of experiments on cats, Hatcher and Brody (7) and, later, Eggleston (8) found the lethal dose of strophanthin to be 0.1 mg. per kilo of

<sup>1</sup> The virulence was such that 0.000001 cc. was fatal in white mice.

body weight. In the present experiments on cats this amount was injected in both the control and the infected animals. In the dogs it was necessary first to estimate the minimal lethal dose (m.l.d.) in normal animals; it was found to be 0.12 mg. per kilo of body weight. Autopsies were performed on all the infected cats and on all cats in the control group which succumbed to the lethal dose of strophanthin. All the dogs, whether normal or infected, were autopsied. In a number of cats and dogs electrocardiograms were taken before infection and also just before injecting strophanthin. A series was also made during the period of injection.

TABLE I.  
*Normal Cats Injected with Strophanthin.*

Date (1914).	Cat.	Sex.	Weight at time of injection.	Stro- phanthin injected.	Amount of m. l. d. + or -.	Result.	Duration of experiment.
			<i>kilos</i>	<i>mg.</i>	<i>mg.</i>		<i>min.</i>
Nov. 3	A	F.	2,200	0.22	L*	D†	52
" 3	B	M.	2,400	0.24	L	D	60
" 3	C	F.	2,100	0.21	L	D	110
" 7	E	M.	3,150	0.315	L	S	—
" 7	F	M.	3,000	0.3	L	D	50
" 7	G	F.	2,500	0.25	L	D	59
" 7	H	F.	2,850	0.285	L	D	70
" 10	I	F.	2,000	0.2	L	S	—
" 10	J	M.	3,650	0.365	L	D	65
" 10	K	F.	2,300	0.23	L	S	—
" 14	L	F.	2,925	0.253	L-0.0395	D	48
" 14	M	F.	4,050	0.405	L	D	63
" 14	N	M.	1,700	0.17	L	S	—
" 20	P	M.	3,300	0.33	L	D	60
" 20	Q	F.	1,975	0.187	L-0.01	D	27
" 20	R	F.	1,700	0.17	L	S	—
" 20	S	M.	1,800	0.18	L	S	—
" 25	T	F.	3,050	0.305	L	D	78
" 25	U	M.	2,800	0.28	L	S	—
" 25	V	M.	2,425	0.2425	L	S	—
" 25	W	M.	2,250	0.225	L	S	—

Total = 21.

Died = 12 = 57.1 per cent.

Survived = 9 = 42.9 per cent.

\* In the tables L represents the estimated lethal dose. L+ or — indicates the amounts more or less than the estimated lethal dose.

† D = died; S = survived.

In the experiments on cats, three groups were studied: a control group and two groups of infected animals. The control group con-

sisted of normal, uninfected animals, each of which received 0.1 mg. of strophanthin per kilo of body weight. This is the amount reported by Hatcher and Brody as the minimal lethal dose. The second group consisted of infected animals. They were otherwise untreated, and served the purpose of observing the course of the disease. The animals in the third group were also infected. Each of these received an amount of strophanthin equal to that given to the first group.

Group I (Table I) formed the control series of experiments. This group consisted of twenty-one cats, all of which were apparently normal and healthy. All received the estimated lethal dose of strophanthin (0.1 mg. per kilo of body weight) in the manner described. Of the twenty-one cats, twelve, or 57.1 per cent, died, while nine, or 42.9 per cent, survived. In the cats that died, the time that elapsed from the commencement of the injection until death occurred ranged from 27 to 110 minutes, the average time being 62 minutes. All the cats received the estimated lethal dose with the exception of animals L and Q. These two died when slightly less than the estimated amount had been injected. The low mortality in this group is a subject which will be discussed later.

TABLE II.

*Cats Infected with Pneumococci, but Receiving No Strophanthin.*

Date of infection (1914).	No. of cat.	Sex.	Type of organism.	Highest temperature be- fore and after infection.	Result.
				° C.	
Nov. 23	34	F.	II	38.5-38.8	D Nov. 26.
" 23	35	M.	II	38.4-39.6	S
" 23	36	M.	II	37.7-39.6	D " 27.
" 23	37	M.	II	38.2-38.6	S
" 24	38*	F.	I	38.2-38.8	D Dec. 14.
" 24	39	F.	I	38.6-39.3	S
" 24	40	M.	I	39.0-39.0	S
" 24	41	F.	I	38.4-39.2	S
" 27	42	M.	II	38.2-39.4	S
" 27	43	M.	II	39.0-39.6	S
" 27	44	M.	II	39.0-39.4	S
" 27	45*	F.	II	37.8-39.0	D " 14.

Died = 16.6 per cent.

\* The two cats, Nos. 38 and 45, which died three weeks after infection, evidently not from pneumonia, but from some intercurrent infection, are not considered in estimating the mortality for the series.

Group II (Table II) comprised twelve cats, each of which was infected by an intrabronchial insufflation of pneumococcus culture. Six of the animals developed very severe symptoms. The other six were only moderately ill. Of the former, one died on the third and one on the fourth day. The symptoms of the disease appeared eight to twelve hours after infection. The animals became listless and prostrated. Almost all showed a rise in temperature ranging from a fraction of a degree to  $3.6^{\circ}$  C. The symptoms of the disease apparently reached their height on about the second or third day after infection. At this point convalescence set in. Practically all the animals lost weight.

Group III (Table III) consisted of forty-three cats, all of which were infected. In seven, the culture used was obtained at autopsy from the heart's blood of infected cats; the rest were infected with organisms obtained in the manner already described. The symptoms of disease corresponded to those described under Group II. Ten cats failed to show a rise in temperature, but the temperature was not taken sooner than twenty-four hours after infection, and it is possible that, had readings been made at an earlier period, a rise might have been observed. A few animals which showed only a very slight reaction to the infecting organisms after the first insufflation, were reinfected. Strophanthin was given as nearly as possible at the height of infection; that is to say, on the second or third day, according to the observations made in Group II. Following the injection, twenty-three cats, or 53.5 per cent, died, and twenty, or 46.5 per cent, survived. The cats that died during or following the injection did so in from 25 to 108 minutes, the average time being 62 minutes. Nine cats succumbed before the injection was completed. They received slightly less than the estimated lethal dose. Three cats, Nos. 17, 48, and 53, through an error in calculation, received slightly more than the estimated lethal dose, but as these cats all survived the error does not affect the results.

A comparison of Groups I and III, the animals in both of which received strophanthin, shows that the mortality in each series, following the injection of the adopted lethal dose of the drug, was practically the same; *viz.*, 57.1 and 53.5 per cent. The course of the disease in the infected animals, however, was short; in many in-

TABLE III.

*Cats Infected with Pneumococci, and Later Injected with Strophanthin.*

Date (1914).	No. of cat.	Sex.	Type of organism.	Weight on day of injection	Stro- phanthin injected.	Amount of m. l. d. + or —.	Re- sult.	Duration of experi- ment.	Highest tempera- ture before and after infection.
				gm.	mg.	mg.		min.	° C.
Oct. 26	1	F.	I	2,250	0.203	L-0.022	D	47	37.8-39.2
" 26	2	F.	I	2,000	0.20	L	S	—	36.2-39.8
" 28	3	M.	II	1,750	0.175	L	D	57	—
" 28	4	F.	II	2,550	0.245	L-0.01	D	62	—
" 29	6	F.	I	2,000	0.15	L-0.051	D	45	36.7-39.0
" 29	7	F.	I	1,300	0.13	L	S	—	36.0-38.0
" 30	9	—	II	2,700	0.27	L	S	—	37.0-38.1
" 30	10	F.	I	2,250	0.225	L	S	—	37.2-37.8
Nov. 2	11	F.	II C*	1,500	0.15	L	D	57	37.8-37.8
" 11	12	M.	II C I	3,100	0.31	L	D	66	38.1-38.6
" 11	13	F.	II C I	1,875	0.1875	L	S	—	38.6-40.0
" 11	14	M.	I	2,575	0.2575	L	D	76	38.8-39.0
" 12	16	F.	II C	3,000	0.30	L	S	—	39.0-39.4
" 12	17	M.	II C	2,200	0.225	L+0.005	S	—	38.8-38.8
" 12	18	M.	II C	2,250	0.225	L	D	88	39.2-40.0
" 13	19	M.	II	3,600	0.3595	L—	S	—	39.0-39.2
" 13	22	M.	II	3,325	0.254	L-0.0785	D	42	39.2-39.0
" 18	23	F.	I	2,425	0.2425	L	D	80	39.1-38.6
" 18	24	M.	I	3,500	0.319	L-0.031	D	62	39.0-38.2
" 18	26	M.	I	2,475	0.2475	L	D	60	38.4-39.4
" 19	15	F.	II C Nov. 10 I 17	2,900	0.183	L-0.107	D	25	38.6-39.6
" 19	27	M.	I	1,700	0.17	L	D	92	39.0-39.8
" 19	28	F.	I	2,225	0.2225	L	D	73	38.4-37.8
" 19	29	F.	I	2,350	0.235	L	D	108	38.2-39.0
" 23	30	F.	II	3,050	0.275	L-0.03	D	48	38.1-38.2
" 23	31	M.	II	3,750	0.375	L	S	—	38.0-40.6
" 23	32	F.	II	2,500	0.25	L	S	—	38.2-39.0
" 23	33	F.	II	2,575	0.2575	L	S	—	38.8-39.6
" 30	48	F.	II	2,700	0.271	L+0.001	S	—	38.8-40.6
Dec. 2	50	F.	II	3,000	0.30	L	D	60	38.5-39.0
" 2	51	F.	II	2,500	0.25	L	S	—	39.0-39.8
" 2	52	F.	II	2,900	0.29	L	D	60	37.5-38.3
" 2	53	M.	II	3,325	0.354	L+0.0215	S	—	38.2-39.3
" 3	55	M.	II	3,850	0.385	L	S	—	39.2-39.0
" 3	56	M.	II	2,950	0.295	L	S	—	38.2-41.2
" 4	57	F.	II	3,100	0.31	L	D	65	38.2-38.8
" 7	62	F.	II	3,000	0.3	L	S	—	38.2-39.4
" 8	63	M.	II	3,400	0.34	L	S	—	38.0-40.0
" 9	64	M.	I	2,500	0.25	L	S	—	37.6-39.2
" 10	65	F.	I	1,550	0.155	L	S	—	38.2-36.6
" 11	66	F.	I	3,050	0.265	L-0.04	D	63	38.0-38.0
" 15	60	F.	II	2,000	0.13	L-0.07	D	37	38.6-38.4
" 18	59	F.	II	1,600	0.16	L	D	46	38.4-39.4

Total = 43.

Died = 23 = 53.5 per cent.

Survived = 20 = 46.5 per cent.

\* C indicates that the culture used was obtained from the heart's blood of a cat previously infected.

stances the animals were not seriously ill, and a number failed to show any increase in temperature, or showed an increase which was comparatively slight. It was considered inadvisable, therefore, to draw conclusions from these experiments. The attempt was accordingly made to enlarge our experience by performing experiments in dogs.

*The Action of Strophanthin in Normal and Infected Dogs.*

The experiments on dogs were undertaken with the expectation that they would show a greater degree of reaction to infection than cats. No standardization of strophanthin in dogs has been made (in so far as could be ascertained), so that it was necessary first to determine the lethal dose of the drug. Experiments were, therefore, carried out on ten dogs (Table IV) to determine the minimum lethal

TABLE IV.

*Dogs Injected with Strophanthin, To Estimate the Lethal Dose.*

Date (1915).	Dog.	Sex.	Weight at time of injection.	Total strophanthin injected.	Strophanthin injected per kilo of body weight.	Duration of experi- ment.	Deviation from average lethal dose.
			gm.	mg.	mg.	min.	per cent
Jan. 20	A	F.	8,000	0.98	0.122	47	2
" 21	B	F.	5,100	0.57	0.111	42	10
" 22	C	M.	7,350	1.07	0.145	110	16
" 25	D	F.	9,000	0.9	0.1	65	17
" 28	E	M.	5,700	0.704	0.123	53	1
Feb. 10	F	F.	5,750	0.75	0.130	95	5
" 10	G	M.	5,000	0.59	0.118	57	5
" 15	H	F.	5,300	0.606	0.114	68	8
" 15	I	F.	4,150	0.55	0.132	90	6
" 16	J	F.	4,750	0.71	0.149	93	20

Average dose per kilo = 0.124 mg.

dose. Before injection the animals were observed for a time under uniform conditions and all were apparently normal and healthy. The injections were made during the months of January and February. The females used were neither pregnant nor lactating. The animals were not fed for a period of eighteen hours preceding the experiment. This short fast was enforced so that the presence of food in the stomach should not influence the weight of the animal, and so that vomiting during the preliminary anesthesia might be prevented.

A fresh alcoholic solution of strophanthin was prepared and the aqueous solution made from this was of a strength such that 5 cc. represented 0.1 mg. of strophanthin. The solution was tested on three cats and was found to have the same effect as had the first stock solution. The lethal dose for these three animals was 0.108, 0.127, and 0.107 mg. per kilo of body weight, respectively.

The injection of strophanthin in dogs was made in a manner identical with that described for cats. The lethal dose for the ten dogs used was found to average 0.124 mg. per kilo of body weight. Death occurred in approximately 60 minutes. The dose ranged from 0.1 to 0.149 mg. per kilo of body weight. Of the ten dogs, seven showed a deviation of 10 per cent or less from the average m. l. d. The remaining three dogs, C, D, and J, gave respectively a deviation of 16 per cent above, 17 per cent below, and 20 per cent above the average. If the three dogs showing the greatest variation be deducted from the series, the result is not materially altered and the m. l. d. still remains 0.12 mg.

TABLE V.

*Dogs Infected with Pneumococci and Later Injected with Strophanthin.*

Date (1915).	No. of dog	Sex.	Type of organism.	Weight before infection.	Weight at time of injection.	Total strophanthin injected.	Strophanthin injected per kilo of body weight.	Duration of experiment.	Deviation from average lethal dose.	Highest temperature before and after infection.
				gm.	gm.	mg.	mg.	min.	per cent	°C.
Jan. 15	1	F.	II	9,000	7,900	1.237	0.137	108	10	38.8-39.2
" 18	3	F.	II	8,150	7,300	1.105	0.151	85	20	39.0-40.9
" 26	4	M.	II	9,400	8,800	1.146	0.130	78	4	39.8-39.5
Feb. 1	5	M.	II	8,500	7,800	0.82	0.105	55	16	39.8-39.5
" 3	6	M.	II	9,000	7,900	0.88	0.111	73	11	39.6-39.2
" 4	8	F.	II	8,300	8,050	1.01	0.125	95	0	39.8-39.6
" 17	10	F.	II	5,800	5,750	0.68	0.118	73	6	38.2-40.4
" 19	11	M.	II	6,350	6,150	0.614	0.0998	55	20	38.8-39.8
" 24	12	M.	I	8,600	7,500	1.08	0.144	115	15	39.0-39.8
" 26	14	M.	I	8,500	8,250	1.1	0.133	90	6	38.5-41.5

Average lethal dose per kilo = 0.125 mg.

A series of ten experiments was next carried out to ascertain the lethal dose in pneumonic dogs (Table V). These animals were infected by the method described. In the reaction to the infection, the dogs displayed symptoms similar to those described for the cats. They became listless and usually refused to eat. The respirations

appeared to be somewhat labored and were increased in rate. In all the dogs there was a loss in weight, amounting in two to more than 1,000 grams. The temperature following infection usually rose. In seven dogs the increase ranged from a fraction of a degree to  $3^{\circ}$  C. Three dogs, however, failed to show an increase, but in these the temperature was not taken until twenty-four hours after infection with pneumococci. The injection of strophanthin was made during the second twenty-four hours following infection because this was the time when the animals were most severely ill. Lamar and Meltzer (5) and, later, Wollstein and Meltzer (9) also give this time as the period of the maximum reaction. The average amount of strophanthin per kilo of body weight injected was 0.125 mg., which was practically the same as the lethal dose required in the normal dogs. The range of the dosage for the infected dogs varied from 0.099 to 0.151 mg. per kilo of body weight. In six of these dogs the amount of strophanthin injected fell within 11 per cent of the average. The remaining four dogs, Nos. 3, 5, 11, and 12, showed respectively a variation of 20 per cent above, 16 per cent below, 20 per cent below, and 15 per cent above. If the four animals showing the greatest variation are omitted from the series it is found that the average m. l. d. of the remaining six dogs is still 0.12 mg. The time which elapsed between the commencement of the injection and the death of the animal varied from 55 to 115 minutes, the average time being 82 minutes. A comparison, then, of the normal and infected dogs shows the lethal dose of strophanthin in each to be the same.

*Autopsy Findings.*—Autopsies were performed on all the cats and dogs in both the normal and infected series, except on the cats in Group I that survived the injection of strophanthin and on the cats in Group II that survived the infection. Autopsies performed on cats of the control series (Group I) killed by strophanthin showed the lungs to be normal. Two cats of Group II, Nos. 34 and 36, died during the height of the infection. No. 34 showed a massive consolidation of the entire left lung, and also of the middle and posterior lobes of the right. No. 36 showed scattered areas of consolidation throughout all the lobes of both lungs, the left lung being almost entirely consolidated. A well marked fibrinous exudate covered the



pleura and a moderate amount of fluid was found in both pleural cavities. Two other cats belonging to this group died at a period three weeks after infection, but their death was evidently due to an intercurrent disease. Autopsies on these animals showed small, scattered, abnormal areas in both lungs suggestive of an old pneumonic process.

Autopsies were performed on all the animals of Group III. The lungs in each instance showed definite signs of consolidation. The involvement varied in extent from a portion of one lobe to an entire lung. The gross examination showed the area involved to be deeply congested and the overlying pleura to be somewhat dull and lacking in luster. In a number of the animals the pleura showed the presence of a small amount of serofibrinous exudate. Two animals, Nos. 33 and 55, developed a fibrinopurulent pleurisy, and one, No. 33, also showed a well marked pericarditis. The portion of the lung involved was usually firm, inelastic, and friable. On section the lungs were firm; the cut surface was uniformly dark red with a tendency to be dry. On compression, a small quantity of bloody fluid exuded from the surface. Small portions of tissue cut from the consolidated areas sank in water. The gross appearance of the lungs in dogs was similar to that in the cats. Blood cultures were made at the time of autopsy from the heart's blood in fourteen cats; of these, twelve contained pneumococci.

Microscopic examination of the lung tissue showed the alveoli to be filled with an exudate consisting chiefly of leucocytes. A few large lymphocytes, red blood cells, and desquamated epithelial cells were also present. Only small quantities of fibrin were contained in the alveolar exudate. The amount of fibrin found in the pneumonias in cats was slightly more than that found in the dogs. The walls of the alveoli were only moderately congested. The bronchi contained small amounts of exudate, consisting chiefly of red and white blood cells with particles of desquamated epithelium and small amounts of fibrin.

*Electrocardiograms.*—Electrocardiograms were made of ten infected cats, of seven normal dogs, and of all the infected dogs. Non-polarizable electrodes were placed on the right fore and the left hind leg. Control curves were obtained before infection of the

animals, before injection of strophanthin, and also at ten minute intervals after the injection was begun. The principal changes found to follow the injection were increase in the conduction time, alterations in the size and form of the T wave, and the production of extrasystolic irregularities. Changes in the P-R time occurring during the course of the strophanthin injection were seen in four of the ten cats. In these there was an increase of 0.02 of a second or more. In the remaining six cats there was no alteration in the conduction time. Changes in the P-R time were also seen in five of the normal and in six of the infected dogs. In all of these there was an increase of 0.02 of a second or more (Figs. 1 B and 3 C). The remaining dogs of both groups showed no important change in the P-R time. Blocking of the auricular impulse occurred in two of the normal and in three of the infected dogs (Figs. 2 B and 3 B).

Alterations in the T wave have recently been shown to occur in man during the administration of digitalis (10). The curves of cats and dogs displayed similar changes (Figs. 4, 5, and 6). Of the ten cats, a negative T wave was present in the control curves of two, and became deeper after the injection (Fig. 5 B). In two others a diphasic T wave became negative, and in a fifth an upwardly directed T wave became markedly diminished in height (Fig. 6). In the remaining five cats, the strophanthin produced no demonstrable change in this wave. In two of the seven normal dogs, the T wave in the control electrocardiogram was positive. After injection it became diphasic in one, and negative (Fig. 1 B) in the other. The remaining five dogs all showed negative T waves in the control curve. On the form of these strophanthin appeared to have no influence. In the ten infected dogs changes occurred more often. In six animals the T wave was positive in the control curve and became negative under the influence of strophanthin (Figs. 3 B and 3 C). In the seventh a negative T wave became diphasic. In the remaining three dogs negative T waves in the control curve became deeper under the influence of the drug (Fig. 4 B). Changes in the T wave were observed, therefore, five times in ten cats, and in twelve of seventeen dogs.

Extrasystolic (11) irregularities occurred in all the animals following the injection (Figs. 3 D and 6 B). Several of the cats

which received the average lethal dose of strophanthin and survived showed an irregularity of this type to a marked degree. When electrocardiograms were made of these animals, twenty-four hours later, the heart rhythm was again normal (Fig. 6). In one cat that survived the injection of strophanthin, persistence of a change of shape in the T wave continued for a period of at least forty-eight hours (Fig. 5). In all the dogs and in the cats that died, extrasystoles multiplied in frequency. At the time of death fibrillation of the ventricles occurred.

#### SUMMARY AND DISCUSSION.

The results of these experiments permit a comparison of the action of strophanthin in the normal and the infected animals. In cats the percentage of deaths following the injection of 0.1 mg. of strophanthin per kilo of body weight was the same in normal and in pneumonic cats. The number of recoveries in this series was larger than was expected. This result was due to the fact that the dose injected was not the lethal dose, but the average lethal dose (0.1 mg.) determined by Hatcher and Brody (7) and by Eggleston (8). The doses which the latter actually injected ranged from 0.085 mg. to 0.16 mg.; from these the average minimal lethal dose was calculated. In adopting the average dose as the standard one to inject, those of our cats that required more than 0.1 mg. naturally survived. The death rate was therefore low. The plan employed in dogs differed from that used in cats. A lethal dose was injected in each dog. Death occurred when an average of 0.12 mg. of strophanthin per kilo of body weight was injected. The same dose was required in normal and pneumonic dogs. The effect of strophanthin in both groups of infected and non-infected cats and dogs is, therefore, identical.

Whether the uniformity of strophanthin action in the two experimental groups may serve as the basis for assuming a like uniformity of action in normal individuals and in pneumonia patients is a subject which requires further analysis. The difficulty in transferring the experimental results to patients lies in the question of whether the type of pneumonia produced in animals is the same as that found

in man. Clinically, the two diseases present both resemblances and differences. The animals become definitely ill and show the symptoms already described. The illness, however, is of short duration and apparently reaches its height in the majority of animals in twenty-four to seventy-two hours. Before the expiration of this time, the temperature frequently returns to normal. Many of the infected animals, when they survive, recover in three to five days. The mortality in dogs infected with pneumococci is given by Lamar and Meltzer as 16 per cent. In the present series of twelve infected cats, the mortality was also 16 per cent. These findings differ from human pneumonia in the following particulars: The infection is not so severe; the temperature, though elevated at first, soon falls; the duration of the disease is short; and convalescence is rapid. The mortality is slightly lower. Musser and Norris (12) give the human mortality at 21.06 per cent. Pathologically the two diseases also show differences. The gross appearance of the lungs is not dissimilar, but in the animals the consolidated portions are somewhat dry and they fail to show a stage of gray hepatization (5). The amount of fibrin present is small. There is comparatively slight congestion of the alveolar walls and of the walls of the bronchi.

The relation of experimental pneumonias to the human disease has been discussed by a number of investigators. Almost all believe that the two types are similar, if not identical. Among the first to express this opinion was Sternberg (13); and later Gamaléia (14), Prudden and Northrup (15), Kinyoun and Rosenau (16), Wadsworth (17), Lamar and Meltzer (5), Wollstein and Meltzer (9) coincided with his view. Lamar and Meltzer, especially, have insisted on the identity of the two processes. On the other hand, Welch (18) in his study of experimental pneumonia, says: "Many inoculations of cultures of virulent pneumococci into the trachea and lungs of dogs have been made in my laboratory by Dr. Canfield and myself, but in no instance were we able to produce an inflammation of the lungs which we were willing to identify with acute lobar pneumonia as found in human beings." But he adds that, in the majority of experiments, there was no demonstrable consolidation and that pleurisy and more or less extensive areas of pneumonia were produced only in a few animals.

The inference consequently cannot be drawn that an effect obtained with strophanthin in the experimental disease may be anticipated in man. The striking similarity in action in infected and uninfected animals renders it likely, however, that the usual action of the drug in man may be expected in the presence of pneumonia. We have accumulated evidence, to be published later, which shows that this action actually takes place in the human disease. As far as evidence obtained electrocardiographically is concerned, our experiments show that strophanthin causes the same electrical changes in the heart when the animals are infected as it does under normal conditions.

#### CONCLUSIONS.

1. When a like amount of strophanthin is injected intravenously, the mortality is the same in both normal cats and in cats suffering from experimental pneumonia.
2. The minimum lethal dose of strophanthin is the same in normal dogs and in dogs suffering from experimental pneumonia.
3. The presence of an acute infection in these animals does not interfere with the action of strophanthin on the heart.
4. Electrocardiographically the changes occurring in the heart's action when strophanthin is injected are found to be similar in normal and in infected animals.
5. The identity of strophanthin action in infected and normal animals renders it probable that a like similarity may be anticipated in man, under normal conditions and in pneumonia.

#### BIBLIOGRAPHY.

1. Mackenzie, J., *Diseases of the Heart*, 3d edition, London, 1913, 379.
2. Gibson, G. A., *Acute Pneumonia: Its Prognosis and Treatment*, *Glasgow Med. Jour.*, 1911, lxxv, 321.
3. Meyer, A. W., *Die Digitalis-Therapie ihre Indikationen und Kontraindikationen*, Jena, 1912.
4. Gunn, J. W. C., *The Influence of Temperature on the Action of Strophanthin on the Mammalian Heart*, *Jour. Pharmacol. and Exper. Therap.*, 1914, vi, 39.
5. Lamar, R. V., and Meltzer, S. J., *Experimental Pneumonia by Intrabronchial Insufflation*, *Jour. Exper. Med.*, 1912, xv, 133.
6. Dochez, A. R., and Gillespie, L. J., *A Biologic Classification of Pneumococci by Means of Immunity Reactions*, *Jour. Am. Med. Assn.*, 1913, lxi, 727.

7. Hatcher, R. A., and Brody, J. G., Biological Standardization of Drugs, *Am. Jour. Pharm.*, 1910, lxxxii, 360.
8. Eggleston, C., Biological Standardization of the Digitalis Bodies by the Cat Method of Hatcher, *Cornell Univ. Med. Coll. Bull.*, 1914, iii, pt. 3, article 10; reprinted from *Am. Jour. Pharm.*, 1913, lxxxv, 99.
9. Wollstein, M., and Meltzer, S. J., Experimental Bronchopneumonia by Intra-bronchial Insufflation, *Jour. Exper. Med.*, 1912, xvi, 126.
10. Cohn, A. E., Fraser, F. R., and Jamieson, R. A., The Influence of Digitalis on the T Wave of the Human Electrocardiogram, *Jour. Exper. Med.*, 1915, xxi, 593.
11. Rothberger, C. J., and Winterberg, H., Studien über die Bestimmung des Ausgangspunktes ventrikulärer Extrasystolen mit Hilfe des Elektrokardiogramms, *Arch. f. d. ges. Physiol.*, 1913, cliv, 571.
12. Musser, J. H., and Norris, G. W., Lobar Pneumonia, in Osler, W., and McRae, T., A System of Medicine, Philadelphia and New York, 1907, ii, 537.
13. Sternberg, G. M., The Pneumonia Coccus of Friedländer (*Micrococcus Pasteuri*, Sternberg), *Am. Jour. Med. Sc.*, 1885, N. S., xc, 106.
14. Gamaléia, M. N., Sur l'étiologie de la pneumonie fibrineuse chez l'homme, *Ann. de l'Inst. Pasteur*, 1888, ii, 440.
15. Prudden, T. M., and Northrup, W. P., Studies on the Etiology of the Pneumonia Complicating Diphtheria in Children, *Am. Jour. Med. Sc.*, 1889, N. S., xcvi, 562.
16. Kinyoun, J. J., and Rosenau, M. J., Infections Caused by Pneumococcus, *Rep. Superv. Surg. Marine Hosp.* 1896-97, Washington, 1899, 762.
17. Wadsworth, A., Experimental Studies on the Etiology of Acute Pneumonitis, *Am. Jour. Med. Sc.*, 1904, N. S., cxxvii, 851.
18. Welch, W. H., The *Micrococcus lanceolatus* with Especial Reference to the Etiology of Acute Lobar Pneumonia, *Bull. Johns Hopkins Hosp.*, 1892, iii, 125.

#### EXPLANATION OF PLATES.

In the following electrocardiograms leads were taken from the right fore to the left hind leg. In all figures the divisions of the abscissæ equal 0.04 of a second, the divisions of the ordinates equal  $10^{-4}$  millivolts.

#### PLATE 74.

FIG. 1, A and B. These curves were taken from Normal Dog B. In A, made immediately before the injection of strophanthin, the P-R interval is 0.07 to 0.08 of a second. The T waves are prominent and upwardly directed. In B, taken 25 minutes after the injection was begun and when 0.43 mg. of strophanthin had been injected, P-R time increased to 0.11 or 0.12 of a second. The T wave is negative. The time since the commencement of the injection and the amount of strophanthin injected are given except in Fig. 3. In the cat curves the time only is indicated.

FIG. 2, A to C. Curves taken from Normal Dog C. Curve A was taken before the injection of strophanthin. The rhythm is regular. The P-R interval is 0.08 to 0.09 of a second. The T waves are negative. Curve B was made 65

minutes after the injection was begun; strophanthin 0.76 mg. had been injected. Complete dissociation between auricles and ventricles is shown. The rate of the auricles is much reduced. Curve C was made 20 minutes later than B; strophanthin 0.85 mg. had been injected. It shows a succession of ectopic ventricular beats. Two distinct types are seen. In the first and last parts of the curve the impulses arose in the wall of the right ventricle; those between, in the wall of the left ventricle. The latter show considerable variation. Auricular complexes cannot be distinguished.

## PLATE 75.

FIG. 3, A to D. These curves were taken from Infected Dog 1. Curve A was made before the injection of strophanthin. The P-R time is 0.10 of a second. The T wave in some of the complexes is flat, in others it is composed of two parts, a low upwardly directed portion and a small negative wave. Curve B was taken 65 minutes after the injection of strophanthin was begun. The P-R time is lengthened to 0.15 of a second. The T waves consist of three parts: first, a gradual upward rise following the S wave; second, a rather sharp downward deflection; and third, an upward rise. Every third auricular impulse is blocked. Curve C was taken 10 minutes after Curve B. The complexes are normal. The P-R time is 0.15 of a second. The ventricular complex is similar to that seen in Fig. 2 B, the principal change being in the T wave. Ventricular extrasystoles occur which are probably of left ventricular origin. The T wave accompanying these is positive. Curve D was taken 25 minutes later than C; strophanthin 1.09 mg. had been injected. No auricular waves appear. In each pair of ectopic ventricular beats the first member has its origin in the left ventricle, and the second in the right ventricle. The third pair forms an exception; here the order is reversed.

FIG. 4, A and B. These curves were taken from Infected Dog 11. Curve A was made before the injection of strophanthin. The P-R time is 0.08 of a second. The T wave is made up of three parts, a small positive wave following directly on the S wave, a small negative, and a small positive wave. Curve B was made 15 minutes after the injection of strophanthin was begun; 0.24 mg. had been injected. The P-R time is unchanged. The T wave is composed of two parts, a negative portion of considerable depth and a small upward part.

## PLATE 76.

FIG. 5, A to D. These curves were taken from Infected Cat 63. The animal survived the injection of strophanthin. Curve A was made before the injection. In the earlier portion of the curve the P wave has a normal relation to the R wave; later it gradually merges into the R wave until it disappears. The excursion of R is now increased and S is decreased. The T wave consists of two portions, a flat or upwardly directed portion and a small negative wave. Curve B was taken 13 minutes after the injection of strophanthin was begun. The P-R time is 0.07 of a second. The T wave consists of two portions, a flat or upwardly directed portion and a negative part of much greater extent than in Curve A. Curves C and D were taken 24 and 48 hours after the injection of strophanthin, respectively. In both curves the P wave is absent and in this

respect they resemble the last portion of Curve A. A negative T wave has persisted in C and D, though in the latter T wave is more like the form seen in Curve A.

FIG. 6, A to C. These curves were taken from Infected Cat 64. This animal survived the injection of strophanthin. Curve A was made before the injection. The heart rhythm is normal. The P-R time is 0.07 of a second. The T wave is positive. Curve B was taken 33 minutes after the injection of strophanthin was begun. It shows a completely irregular heart action. No P waves are discernible. The ventricular complexes are ectopic, have various forms, and have their origin at several points in the walls of the right and left ventricles. Curve C was taken 24 hours later and shows the return of the heart to a normal rhythm. The P-R time is the same as in Curve A. The T waves are positive.





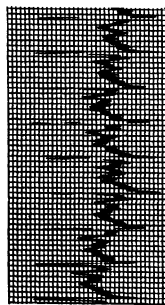


Fig 1A. Dog B.

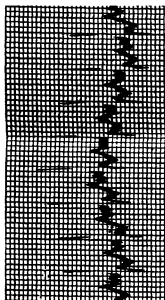


Fig 1B. 25 min. 0.45 mgm.

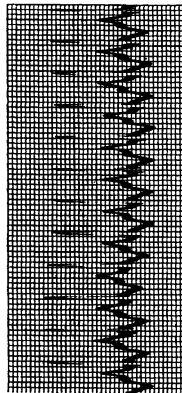


Fig 2A. Dog C.

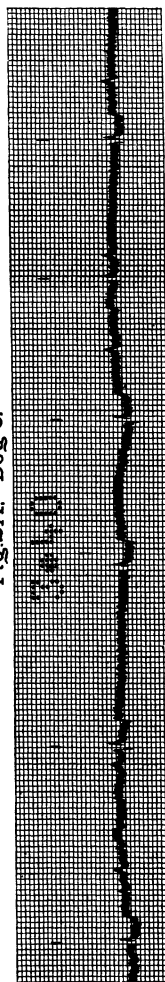


Fig 2B. 65 min. 0.76 mgm.



Fig 2C. 85 min. 0.85 mgm.

(Translation: Action of Lethal Dose of Strophanthin in Animals.)

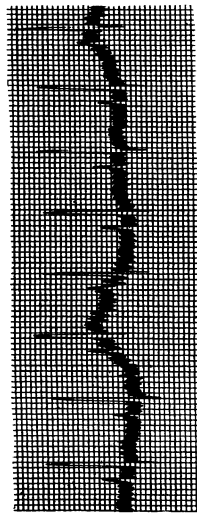


Fig. 3A. Dog 1.

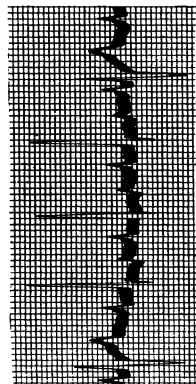


Fig. 3C. 75 min.

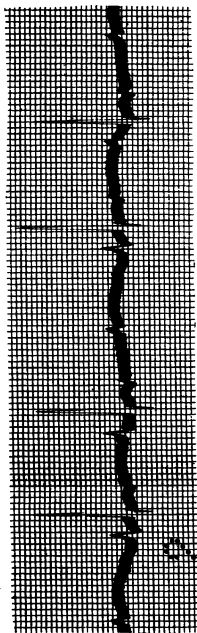


Fig. 3B. 65 min.

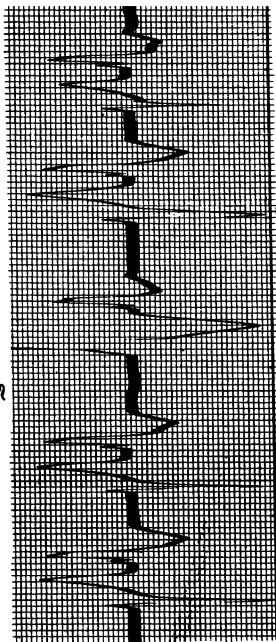


Fig. 3D. 100 min.

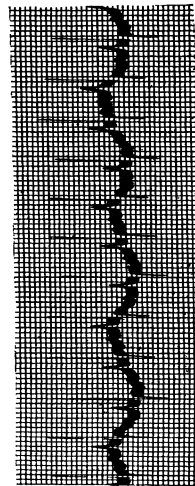


Fig. 4A. Dog 2.

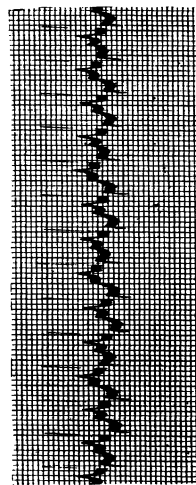


Fig. 4B. 15 min. 0.24 mgm.

Janieson: Action of Lethal Dose of Strophanthin in Animals.

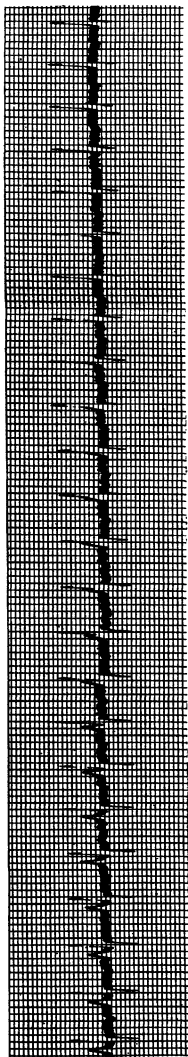


Fig. 5A. Cat 63.

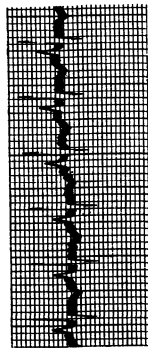


Fig. 5B. 13 min.

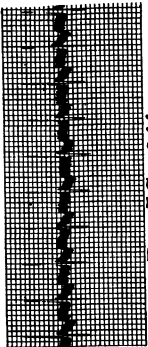


Fig. 5C. 24 hrs.

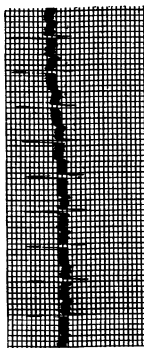


Fig. 5D. 48 hrs.

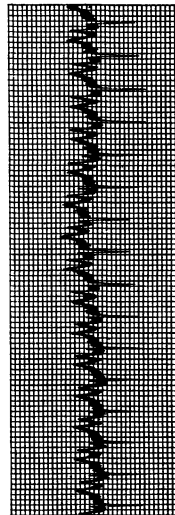


Fig. 6A. Cat 64.

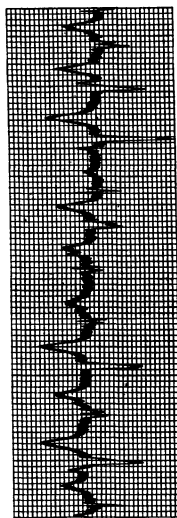


Fig. 6B. 33 min.

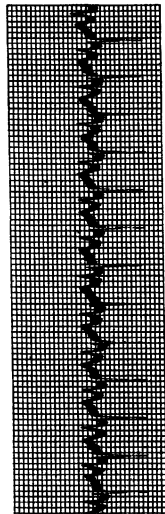


Fig. 6C. 24 hrs.



## NOTE CONCERNING EXERCISE IN THE TREATMENT OF SEVERE DIABETES.

By FREDERICK M. ALLEN, M.D.

*(From the Hospital of The Rockefeller Institute for Medical Research.)*

Authorities on diabetes have agreed that muscular exercise is a useful means of increasing tolerance in cases of mild or moderate diabetes, but is inadvisable in the most severe cases, where it may increase glycosuria, exhaust strength, and even send the patient into coma. It has been a stringent rule that severe diabetics should be guarded against all excessive exertion and fatigue.

Since the changes in diabetic treatment now transform severe into mild cases as far as freedom from glycosuria and acidosis is concerned, it was considered worth while to investigate whether such patients might also react to exercise in the same way as mild cases. Tests were made first on diabetic dogs, with a known constant limit of tolerance for carbohydrate or protein. It was found that vigorous exercise on the treadmill markedly raised the tolerance of such animals, as judged by the sugar in both urine and blood. In some experiments, dogs which for months past had regularly shown glycosuria whenever they were given 100 grams of bread, on exercise became able to take 200 grams of bread as a regular daily ration without glycosuria.

The tests with patients are more recent, but the results thus far appear sufficiently favorable to warrant recommending exercise as an addition to the treatment. Just how early the exercise is begun may vary with individual patients. It seems possible that the stronger patients may shorten their initial fast by this means if desired. As soon as the first few days of treatment have markedly reduced glycosuria and ketonuria, the dangers previously feared from over-exertion are apparently removed. Naturally, some of the severest cases are too weak for exercise at first, but it is begun

as early in the period of dieting as practicable, and generally the weak patient is able to do more than he or his physician supposed. In suitable cases the blood-sugar may be found to fall rapidly during a half-hour or hour of lively exercise. Early in treatment, or in the more severe cases it may rise thereafter; but often it will continue to fall after the exercise is ended, and remain for some time at a lower level. In a patient free from glycosuria with persistent hyperglycemia, one fast-day with exercise may reduce the blood-sugar as much as several fast-days without exercise. If glycosuria is produced in a patient by adding either carbohydrate, protein, or fat to the diet, it is frequently possible to abolish this glycosuria by exercise while continuing the increased diet.

It seems advantageous to give exercise especially after a meal containing carbohydrate or other food tending to produce glycosuria, although, when patients are able, they exercise at all times of the day. At present, short periods of vigorous exercise with rests between are preferred to long slow walks, which might tire the patient even more. The exercises now suggested are running up and down stairs, jumping rope, throwing a heavy "medicine ball," and turning somersaults. Tennis and other hard games should probably be beneficial. At first, precautions may be taken against the nervousness and sleeplessness sometimes caused by over-weariness in weak patients. Otherwise, patients are worked right up to the limit of their strength, somewhat like athletes in training. In regard to the reduction in weight which has been advised in the fasting treatment, the question has arisen as to what kind of tissue it is desired to reduce. The answer seems to be that it is desirable to reduce fat and reserve tissues, and build up active muscular tissue. With this end in view, the emaciated, flabby-muscle diabetic is turned into an athlete as far as practicable. During exercise, no attempt is made to shield the patient against excitement, invigorating cold, or any similar influences previously dreaded in diabetes. It is hoped that an end may be put to the period of pale, feeble diabetics, dressed in double underwear while hugging the radiator and growing more neurasthenic all the time. Some of the exercises above mentioned are planned to shake up these patients and break up their former bad habits, both physically and psychically. The

patients feel much better; they are kept occupied during the day, and sleep well at night. Hunger may be partly satisfied by vegetables and bran cakes, but in general exercise has increased appetite less than it has increased the power to satisfy appetite. The patients can take a somewhat more liberal diet, and enjoy the possession of somewhat greater weight and strength; but the increase of weight in this instance consists of muscle not fat.

It is hoped that this addition to the treatment of severe diabetes will prove of especial value to children, to patients with persistently low tolerance, and perhaps to some of that class previously so hopeless, viz. tuberculous diabetics. For the ordinary type of patients it may be a means of getting results somewhat more quickly and thoroughly, and leading to a higher degree of both comfort and usefulness. The value of exercise is strictly limited. It cannot raise tolerance very high, and it is not equal to the dietary régime in importance. Results will be unfortunate if it is used merely as a means for shortening the hospital care of the patient, or for building up weight and strength at the cost of more important considerations. The radical and permanent control of the diabetes is the essential matter, and is to be judged by such things as glycosuria, acidosis and blood-sugar, not by a temporary sense of well-being. A stern program of fasting, low diet, and reduction of weight is still necessary as before, but it is hoped that results may be more beneficial with the use of exercise as an additional detail of the treatment. The experiments with exercise are still in progress and will be published in detail later, together with a discussion of their theoretical bearings.



## THE ABDERHALDEN REACTION.

By DONALD D. VAN SLYKE, MARIAM VINOGRAD-VILLCHUR,  
AND J. R. LOSEE.<sup>1</sup>

(*From the Hospital of The Rockefeller Institute for Medical Research and the Lying-In Hospital of New York.*)

(Received for publication, September 29, 1915.)

Despite the tremendous amount of work which has been inspired by Abderhalden's idea of protective ferments, there has as yet been no indication of an approach to universal agreement concerning their specificity or utility for diagnostic purposes. The value of the "Abderhalden reaction," even for the detection of pregnancy, to say nothing of the diagnosis of less readily defined pathological conditions, is as much a matter of contention at present as when attempts to utilize the reaction first became general. The number of papers which have appeared both for and against the reliability of the reaction is so great that we shall attempt no review of the literature, most of which is cited in the bibliography of the latest edition of Abderhalden's "Abwehrfermente."

The present work was undertaken in the hope of providing for the measurement of serum protease a quantitative method sufficiently simple, accurate, free from subjective influence, and specific for proteolysis to afford definite conclusions concerning at least the facts of the Abderhalden reaction.

In attempting to ascertain the latter we have not investigated any of the applications of the Abderhalden reaction to pathology, but have confined ourselves to a study of pregnant compared with normal sera; because with these, according to the work of Abderhalden, one should certainly expect the most clear cut and reliable results.

<sup>1</sup> Preliminary reports of the work here presented have been published in *Proc. Soc. Exper. Biol. and Med.*, Van Slyke, D. D., and Vinograd, M., 1914, xi, 154; Van Slyke, D. D., Vinograd, M., and Losee, J. R., *ibid.*, 1915, xii, 166.

There is, furthermore, no chance for error in final confirmation of the diagnosis.

The method in most general use has been Abderhalden's "dialysis method," which, as described in the "Abwehrfermente," is so well known that description here is unnecessary. The fact that the method is not quantitative, and that even after the procedure has been successfully carried through all the preliminary details the final decision as to whether the result is positive or negative is based on the matching of colors, leaves the results peculiarly open to subjective influence on the part of the manipulator. Aside from this, the points for the possible introduction of errors are so numerous that it appeared possible, as Abderhalden has contended, that every failure by others to get good results with the dialysis method has been due to errors in technique.

The other method which has been chiefly utilized by the Abderhalden school has been the optical one, in which serum is incubated with peptone substrate, and the change in rotation observed in a polariscope. As the nature and optical rotation of the products are unknown, however, it is difficult to give a definite interpretation to the results. A technical difficulty lies, furthermore, in the slightness of the changes frequently observed. Being only a few hundredths of a degree, they often lie barely outside the possible limits of error in reading the instrument.

In the attempt to obtain quantitative results, Michaelis<sup>2</sup> utilized the familiar principle of measuring proteolysis by determining the non-coagulable nitrogen. After the serum had been incubated with substrate the proteins were precipitated by Michaelis and Rona's well known colloidal ferric hydrate method, and the amount of nitrogen in the filtrate was determined. Michaelis himself found the results by this method of as little diagnostic value as those he obtained by the dialysis procedure, but the colloidal iron method was later adopted by Abderhalden with excellent results. Other modes of coagulation have since been used, with varying success.

Another attempt to make the reaction quantitative was made during the past year by Thar and Kotschneff,<sup>3</sup> utilizing the amino nitrogen method devised by one of the writers. The serum was incubated with placenta peptone, and the resulting increase in amino nitrogen in the mixture determined. There was no

<sup>2</sup> Michaelis, L., and Lagermarck, L. v., *Deutsch. med. Wchnschr.*, 1914, xl, 317.

<sup>3</sup> Thar, H., and Kotschneff, N., *Biochem. Ztschr.*, 1914, lxiii, 483; 1915, lxix, 389.

definite difference between the results from normal sera and those from pregnant sera. In other experiments, however, in which placenta and serum were incubated in dialyzing thimbles and the amino nitrogen was determined in the dialysate, the results were negative with normal sera, but positive with nephritic as well as with pregnant sera. The protease of pregnant sera did not appear specific for placenta tissue, since carcinoma and lung tissues were also digested.

Recently, on the other hand, Abderhalden has published a series of experiments<sup>4</sup> in which a number of sera, pregnant, normal, and from animals in which specific ferments were supposed to have been generated by injection of proteins, were tested for specific proteases by all of the above methods, as well as the interferometric method of Hirsch, and the same results were obtained without exception by all the different means utilized. In each case where the conditions were such as to indicate according to the theory that a specific protease was to be expected it was found, and in all other cases the substrates were not attacked at all.

As a possible standard method for measurement of serum protease the amino nitrogen determination seemed to us particularly promising for the following reasons: First, it is *quantitative*, and permits accurate results with the small amounts of material available. Second, it is *specific for proteolysis*; it permits one to follow the chemical change which is characteristic of protein hydrolysis; *viz.*, the transformation of non-amino nitrogen in the —CONH— peptide linkings into primary amino nitrogen as these linkings are hydrolyzed with the formation of —COOH and —NH<sub>2</sub> groups.

At first we utilized the simplest possible conditions. The substrate, dried at room temperature at 0.5 mm. pressure over sulphuric acid and then pulverized under sterile conditions, was incubated with the serum. After incubation the mixture was diluted with a volume of water equal to that of the serum, centrifugated, and the amino nitrogen content in 1 or 2 cc. was determined by the micro-amino method of Van Slyke. The results were compared with those from control portions of serum similarly treated in the absence of substrate.

Further experience showed that it was advantageous to remove the proteins before determining the amino nitrogen. The albumin and globulin of the serum contain, even when undigested, 8 and 5 per cent respectively of their total nitrogen in the form of free

<sup>4</sup> Abderhalden, E., *Fermentforschung*, 1914, i, 20.

amino groups,<sup>5</sup> representing the  $\omega$ -amino groups of the lysine molecules in the proteins. This amino nitrogen of the intact serum proteins amounted to three or four times the maximum which we observed formed as the result of the digestion with the substrate. The percentage accuracy with which the increase could be determined was consequently somewhat diminished by the amount of amino nitrogen present besides that due to digestion. Also the  $\omega$ -amino group of lysine requires 15 to 30 minutes, according to the temperature, to react completely, while the  $\alpha$ -amino groups liberated during digestion react in 3 to 4 minutes. In 5 minutes a definite proportion, from 80 to 90 per cent of the  $\omega$ -nitrogen, according to the temperature, reacts with nitrous acid under the conditions of the determination; so that, by running the control determinations at the same temperature and for the same definite reaction period of 3, 4, or 5 minutes, increases due to liberation of  $\alpha$ -amino groups by proteolysis can be measured with a fair degree of accuracy.

It is much more satisfactory, however, to remove the undigested proteins before determining the amino nitrogen of the digestion products, and we have found that the Michaelis colloidal ferric hydrate method affords an excellent means for accomplishing the removal. As the results by this technique are more accurate and the percentage increases in amino nitrogen greater than those observed when the proteins are present, it appears worth while to publish only the results obtained by the better method. We may state, however, that those obtained without removal of the proteins were of exactly the same significance as those given below.

#### *Experiments with the Colloidal Ferric Hydrate Method in Preparing Serum for Amino Nitrogen Determination.*

C. G. L. Wolf has already shown that Michaelis' colloidal ferric hydrate method is suitable for removing the proteins from blood in order to obtain a filtrate for quantitative determination of free amino nitrogen.<sup>6</sup> In experiments on Witte peptone and partially digested proteins to be published later we have found, furthermore, that col-

<sup>5</sup> Hartley, P., *Biochem. Jour.*, 1914, viii, 541. Van Slyke, D. D., and Birchard, F. J., *Jour. Biol. Chem.*, 1913-14, xvi, 539.

<sup>6</sup> Wolf, C. G. L., *Jour. Physiol.*, 1914, xlix, 89.

loidal ferric hydrate not only lets all the amino-acids go through into the filtrate, but that it also precipitates none of the intermediary products up to the albumoses, and none of these except some of complexity but little below that of the original proteins (proportion of amino nitrogen was but 6 to 7 per cent of the total in the precipitated albumoses). As the precipitation of the native proteins themselves is complete, colloidal ferric hydrate appears especially well adapted to our purpose.

The following experiments show that closely agreeing results are obtained, even when the conditions of precipitation are not kept at all constant. In each case 2 cc. of normal horse serum, containing 7.5 per cent of protein, were diluted with 20 cc. of water and heated to boiling. The designated amount of Merck's colloidal ferric hydrate (containing 5 per cent of  $\text{Fe}_2\text{O}_3$ ) was then added drop by drop. After a few seconds' boiling the magnesium sulphate, a solution made by dissolving  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  in an equal weight of water, was added to coagulate the excess of iron. The solution was then filtered through a folded paper into a 100 cc. Jena glass evaporating dish and washed four or five times with hot water. The filtrate was in every case except No. 7 water-clear. It was evaporated to dryness on the steam bath, the solution being transferred toward the end of the evaporation to a dish of only 4 or 5 cm. diameter. The dry residue was redissolved in 0.5 cc. of water, and transferred to the burette of the micro-amino apparatus. The apparatus had already been charged with nitrous acid and freed from air (first stage of amino nitrogen determination). The dish and burette were then washed with three more portions of 0.3 cc. each of water, and the apparatus was shaken 4 minutes, the determination being completed in the usual manner. The correction for the reagents was 0.040 cc. of gas. The corrected readings of nitrogen gas are given in the following table.

#### *Preparation and Testing of Placenta Substrate.*

We have utilized placentas prepared in three different ways: (1) According to Abderhalden's directions in every possible detail, and preserved in water under toluene. (2) Prepared practically the

TABLE I.

No.	Serum.	Colloidal iron solution.	MgSO <sub>4</sub> solution.	Nature of precipitate and filtration.	Nitrogen gas at 24°, 764 mm., obtained in amino nitrogen determination.
	cc.	cc.	cc.		cc.
1	2	3	1	Filtered clear but slowly. Precipitate bulky, apparently containing large excess of Fe(OH) <sub>3</sub>	0.311
2	2	3	1	"	0.311
3	2	2	0.5	Filtered clear and more rapidly than 1 and 2. Precipitate less bulky	0.304
4	2	1	0.5	Ideal precipitation. Precipitate settled at once. Filtrate came through water-clear, and nearly as rapidly as hot water through an empty filter paper	0.313
5	2	1	0.5	"	0.310
6	2	1	0.5	"	0.306
7	2	0.5	0.5	Too little iron used. Protein not all precipitated. Solution foamed and clogged filter paper. Filtrate showed biuret and Heller's test	

same as (1), but dried at room temperature and 0.5 mm. pressure, pulverized, and preserved dry in sterile bottles. (3) According to the method recently recommended by Pregl<sup>7</sup> as an improvement on Abderhalden's. The Pregl method, like (2), yields dry, pulverized material.

Abderhalden lays especial emphasis on the necessity for the careful preparation of the placenta substrate in a condition free from soluble nitrogen capable of giving the ninhydrin test for amino-acids, free from hemoglobin, but not deprived of the delicate epithelial tissue, which appears to be or to contain the specific substrate attacked by the serum protease. All of the placentas were prepared with the strictest regard to the prescribed precautions. They were brought immediately from the operating room to the laboratory of the Lying-In Hospital, and the washing was begun while they were still warm. All used in the work reported here were prepared after considerable experience had been gained during the preliminary work spent in developing the technique finally adopted for carrying out the entire reaction. Also, one of us, L., had had previous experience in preparing placentas for the Abderhalden reaction.

<sup>7</sup> Pregl, F., *Fermentforschung*, 1914, i, 7.

We believe that our substrates met the requirements cited by Abderhalden as closely as extreme care and a fair amount of experience could render possible.

The placenta tissues were tested at intervals for amino nitrogen by digesting 0.1 gram of dry tissue, or as nearly as could be judged an equivalent of the wet, with 2 cc. of water for 16 hours in the incubator, clearing the mixture with colloidal iron, and determining the amino nitrogen in the filtrate. In no case was a placenta used which yielded more than 0.01 cc. of nitrogen gas.

None of the water extracts showed any trace of hemoglobin. The tissue preparations themselves were quite white.

The sterility, both of the substrates and of all the operations connected with the reaction, was occasionally controlled by plating serum which had been digested with substrate in the usual manner. Most of the plates were sterile after 48 hours' incubation. A few showed isolated colonies. We believe that the influence of bacterial contamination on our results has been excluded.

The Pregl placentas (Class 3) as well as those prepared according to Abderhalden met the tests described above. A large amount of the tissue was lost as the result of the harsh mechanical treatment involved in the Pregl preparation, and the portion which was left both looked and behaved chiefly like resistant connective tissue. The results obtained with it were different from those obtained with the Abderhalden substrates only in that the Pregl placentas gave smaller amounts of digestion products with both normal and pregnant sera.

*Details of Preparation of Placenta.*—About 5 minutes after delivery, the placenta was placed in a basin with physiological salt solution and kneaded therein for the purpose of forcing the blood out of the vessels. Fresh portions of salt solution, alternated with distilled water, were added, about 2 liters being used for each washing. This kneading and washing continued until the placenta was nearly decolorized. After several washings, the placenta appeared as a pink colored mass, and the most difficult part of the work was to remove this color. This required many washings. Toward the end of the washing process, the blood vessels were carefully cut away from the placental tissue with small scissors. After the tissue was freed from blood vessels the washing was continued for some time, until the pink color disappeared entirely. The parts of the tissue which were not sufficiently decolorized were separated and thrown away. The whole washing period took from 3 to 4 hours.

Not all the placentas could be washed satisfactorily, as some of them retained some pink color even after numerous washings. Only the entirely decolorized placentas were used.

The coagulation of the proteins of the placenta and the extraction of amino-acids were accomplished by boiling the tissue in water slightly acidified with acetic acid. The amount of water used each time was about ten times the amount of placental tissue, and the boiling was repeated five or six times. The first time the boiling was continued for 30 minutes; the subsequent boilings were kept up for 5 minutes each.

The placenta prepared by the above method, together with some of the water used in the last boiling, was placed in a glass-stoppered bottle of such size that it was filled to about three-fourths of its capacity, and then sufficient toluene was added to completely fill it. The bottles were kept in an ice chest.

In Placentas 1 to 7 only, which were prepared in dry powder form, the Abderhalden method was varied as follows. The time of the washing process was considerably shortened by the use of 1 per cent solution of sodium citrate for the first washing. This prevented the blood from coagulating. From this stage on the usual method of washing was followed. After the last boiling, the water was carefully decanted and the tissues were rapidly dried in a desiccator under 0.5 mm. pressure, then pulverized under sterile conditions. The powder was kept in a sterile glass-stoppered bottle at room temperature.

In the preparation of the Pregl placentas Pregl's directions were followed entirely.

### *Details of the Serum Test.*

The blood was allowed to stand for 3 to 4 hours in the sterile tube into which it had been drawn, in order to allow time for the clot to contract. The serum was then decanted off, centrifugated, and the clear serum removed with a pipette. The serum was then submitted to a second centrifugation, in order to make certain that all formed elements were removed. Repeated microscopic examinations failed regularly to reveal cells of any type in the serum after the second centrifugation. It was also free from hemoglobin.

Of the clear serum 2 cc. measured with a bulb pipette were placed in a sterile tube with the substrate and covered with a layer of toluene 2 or 3 cm. deep. When dry pulverized placenta was used as the substrate 0.100 gram was weighed out on a sheet of aluminum foil which had immediately before been sterilized in a flame. When wet substrate was used, preliminary tests were made to ascertain the approximate bulk of the substance which contained 0.1 gram of dry material, and this amount was afterwards taken, as nearly as could be judged by the eye, for incubation with the serum. Care



was taken that no pieces of substrate adhered to the sides of the tube above the toluene. Controls were prepared in the same way, except that no placenta was added. The tubes were stoppered with sterile cotton and placed in the incubator at 37° for 16 hours.

At the end of this time the contents were washed into a Jena beaker of 100 cc. capacity, about 20 cc. of water being used in the transfer. The mixture was heated until it began to boil, then Merck's colloidal ferric hydrate (containing 5 per cent of  $\text{Fe}_2\text{O}_3$ ) was added from a pipette drop by drop, 1 cc. for the controls, 2 cc. for the mixtures containing substrate. The liquid was boiled for 15 seconds or longer after all the colloidal ferric hydrate had been added, then 0.5 cc. of a 1 : 1 solution of crystalline magnesium sulphate was added, and the boiling continued for another fraction of a minute. The precipitate was then allowed to settle, and the solution decanted through a small folded filter into a Jena glass evaporating dish of about 100 cc. capacity. The precipitate was then washed several times, partly by decantation, partly on the filter, with hot water, the volume of the portions being so regulated that the combined filtrate and washings nearly filled the evaporating dish. The precipitate was granular, and could be washed so rapidly that both coagulation and washing were easily completed in five minutes. The washing removes every measurable trace of uncoagulated amino nitrogen. We repeatedly submitted precipitates to a repetition of the washing, evaporated the filtrates from the second washings separately, and attempted to determine amino nitrogen in them. The results were also negative.

The filtrates obtained as above were evaporated on the steam bath until they were dry, or only a few drops of water remained. Standing on the bath for an hour after they had become dry did not appear to affect the amino nitrogen content of the residues, but the dishes were regularly removed from the bath within at most a half hour after the water had evaporated, and usually within a few minutes.

The quantitative removal of the redissolved residue to the micro-amino apparatus was rendered more easy by transferring the solution, after it had been concentrated to a few cc., to a smaller evaporating dish, and completing the concentration in that.

The final residue was redissolved in 0.5 cc. of water, with the aid

of a slender, rubber-tipped stirring rod. The micro-amino apparatus<sup>8</sup> was then charged with nitrous acid and freed from air by two minutes' shaking (first stage of the amino nitrogen determination). The solution from the evaporating dish was then poured into the burette of the amino apparatus and the dish was washed with three successive portions of 0.3 cc. of water each, each portion being so guided by the rod during the transfer to the burette that the inner wall of the latter was washed down around the entire circumference. Each portion of the washing solution was admitted from the burette into the deaminizing chamber, so that the three portions used washed thoroughly both the evaporating dish and the burette, and transferred the entire serum residue to the deaminizing vessel of the amino apparatus. Less than a minute sufficed for the entire transfer, and it was performed in approximately the same time for the residues from the control tube and the tube containing placenta, so that the periods during which each was acted on by the nitrous acid should be as nearly as possible equal. As soon as the transfer was complete the apparatus was shaken for either 4 or 5 minutes, according as the temperature was above or below 20°. The determination was completed in the usual manner, and the volume of nitrogen read off in the gas burette.

Reference to Table I and to the duplicate controls in the tests with human serum shows that the errors accumulated during the entire manipulation seldom caused variation in the final result exceeding 0.01 cc. of nitrogen gas. The amounts of nitrogen gas (corrected for the reagents) from the controls varied from 0.18 to 0.28 cc. The presence of placenta substrate during the incubation caused increases usually between 0.05 and 0.20 cc. and sometimes over 0.25 cc. The changes observed were, therefore, many times greater than the experimental error.

It was thought that submitting the serum residues to acid hydrolysis, thereby changing peptones resulting from digestion of serum or substrate into amino-acids, might, through increasing the volume of gas obtained to measure, make the method still more sensitive. It was found, as a matter of fact, that the increases averaged about

<sup>8</sup> The form of apparatus used was that described in the note following this article.

three times as great as those above mentioned, indicating that the average complexity of the proteolytic products in the colloidal iron filtrate was very roughly approximated, that of tripeptides. The results are given in Table II chiefly because this point may be of interest. For these determinations the residues were taken up in 20 per cent hydrochloric acid instead of water, and were heated in loosely stoppered tubes at 100° for 24 hours to hydrolyze the peptone. The solutions were then evaporated to dryness on the water bath, and used for amino nitrogen determinations. The addition of the hydrolytic treatment to the process, however, increased the error in the controls from 0.01 cc. to about 0.05 cc. of nitrogen gas. Consequently hydrolysis added nothing to the accuracy of the method, and the procedure adopted as the standard is the simpler one outlined in the previous paragraphs.

#### RESULTS.

The nature of the results is so readily apparent from inspection of the tables, and particularly of the charts, that discussion seems unnecessary. The conclusions which appear evident from them are stated in the following summary.

#### SUMMARY.

A simple and quantitative method has been established for measuring by amino nitrogen determination the extent of the proteolysis occurring when serum and substrate are incubated as in the Abderhalden reaction. The mixture after incubation is freed from protein with colloidal ferric hydrate, the filtrate evaporated, and the free amino nitrogen in it determined with the micro-amino apparatus. The increases in amino nitrogen observed when digestion occurs are many times greater than the experimental error of the method; so that it appears possible to rule out the latter as a factor in the results.

As controls, normal, not pathological, sera have been used; although as a point of independent interest, some determinations on pneumonic sera are reported.

Practically every serum, whether from a pregnant or a non-preg-

nant individual, showed protein digestion when incubated with placenta tissue prepared according to Abderhalden. The range of individual variation in proteolytic activity was wide. The range covered by most of the normal sera was, however, identical with that covered by the majority of the pregnant sera. As can be seen by reference to the charts, there is a tendency for the results from the pregnant sera to average somewhat higher than those from non-pregnant. The difference, even in the averages, is not great, however; and the individual variations of both pregnant and non-pregnant sera make the results from both overlap so completely as to render the reaction, even with quantitative technique, absolutely indecisive for either positive or negative diagnosis of pregnancy. The force of this statement is made apparent by even a cursory examination of the charted results.

Further evidence of non-specificity is seen in the fact that carcinoma tissue was digested to about the same extent as was placenta.

It appears that nearly all human sera can digest certain coagulated tissue proteins to some extent, but that the source and significance of the proteolytic agents, and the influences that cause their fluctuation, remain as yet undetermined.

We wish to express our appreciation to Dr. J. W. Markoe, Director of the Lying-In Hospital, for the assistance which he has afforded this work in placing the facilities of the Lying-In Hospital at our disposal; to Dr. C. F. Jellinghaus, to whom we are indebted for a portion of the material used in obtaining the results reported; to Dr. Isaac Levin for prepared carcinoma tissue; and to Dr. Cragin and Dr. Frederick Lyon of the Sloane Hospital for their courtesy in furnishing the material which rendered possible the preliminary work on the methods adopted.

## EXPLANATION OF FIGURES.

The figures present graphically the extent of digestion observed with the different sera and substrates and expressed numerically in the last columns of the tables.

The abscissæ represent values of the difference (cc. of  $N_2$  from 2 cc. serum incubated with placenta) — (cc. of  $N_2$  from 2 cc. serum incubated alone); *i.e.*, the abscissæ give in terms of amino nitrogen the extent of protein digestion caused by the interaction of serum and placenta.

Results from normal male	sera	are indicated by	●
" " " female	" " "	"	●
" " pregnant	sera	" " "	×
" " pneumonic	sera	" " "	□

The results obtained with each placenta are grouped between a pair of horizontal lines, each circle, cross, or square representing the result obtained with the serum of one individual acting on the placenta indicated. (In Table II results with one carcinoma tissue are also given.)

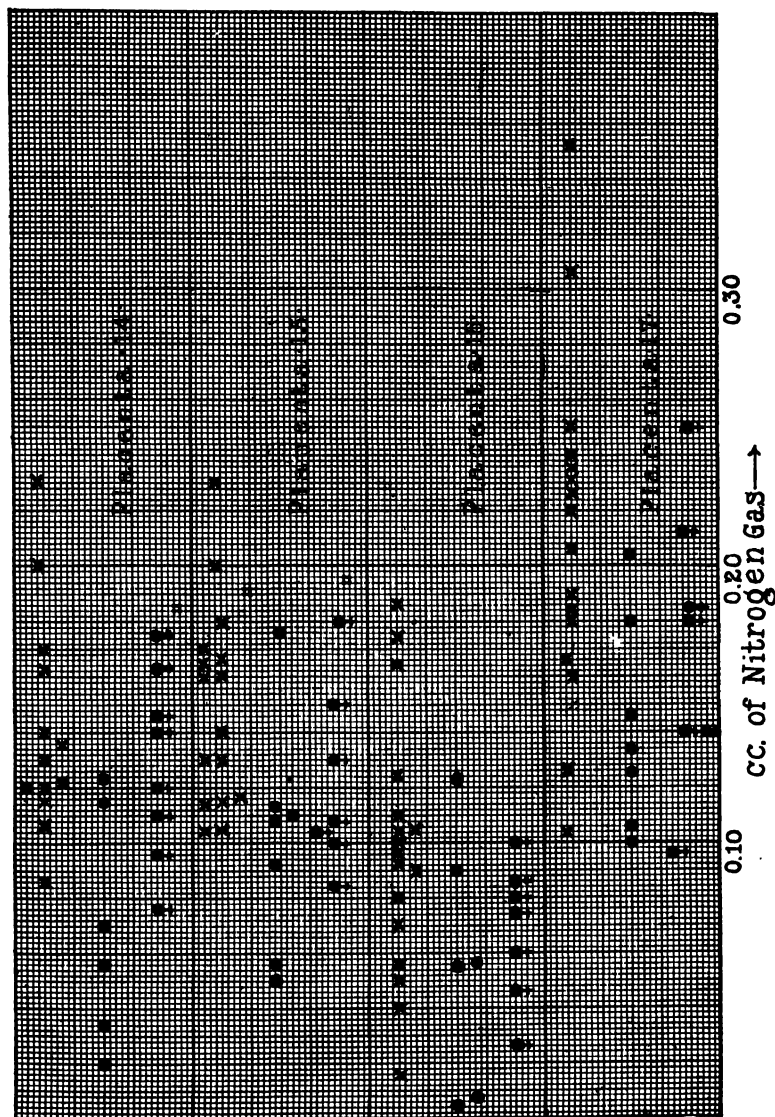


FIG. 1. (For explanation of symbols see p. 470.) Results from placentas prepared according to Abderhalden and preserved under toluene. With a given placenta it is seen that a majority of the results from both pregnant and non-pregnant sera fall within the same limits.

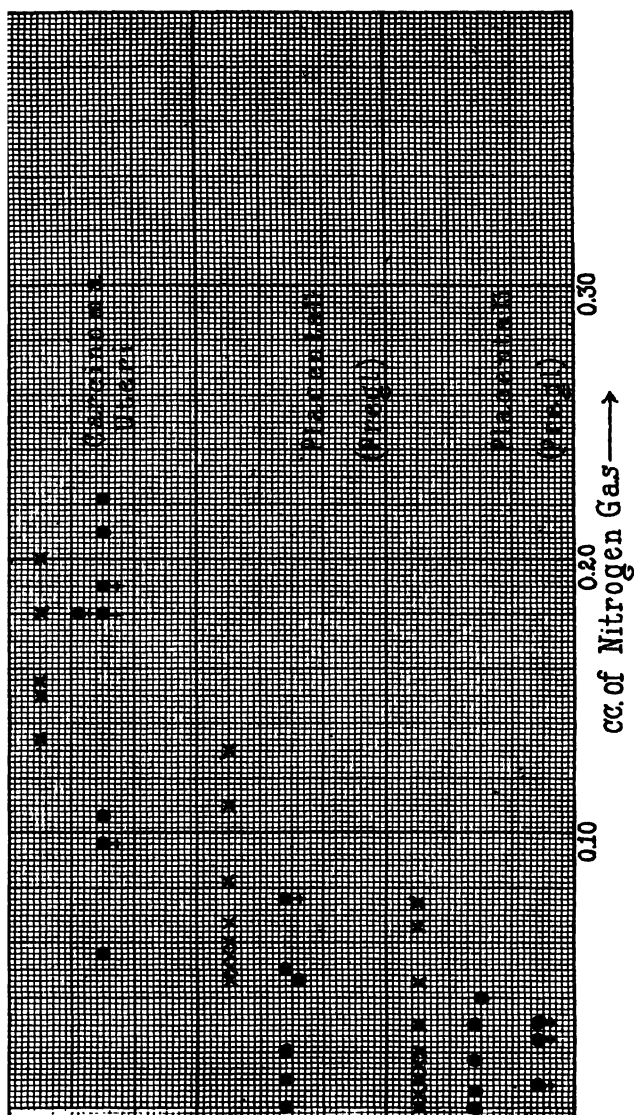


FIG. 2. (For explanation of symbols see p. 470.) Results from carcinoma tissue prepared according to Abderhalden and preserved dry, and from placentas prepared according to Pregl. Less digestion is observed with Pregl than with Abderhalden placentas, but the same non-specific results are obtained.

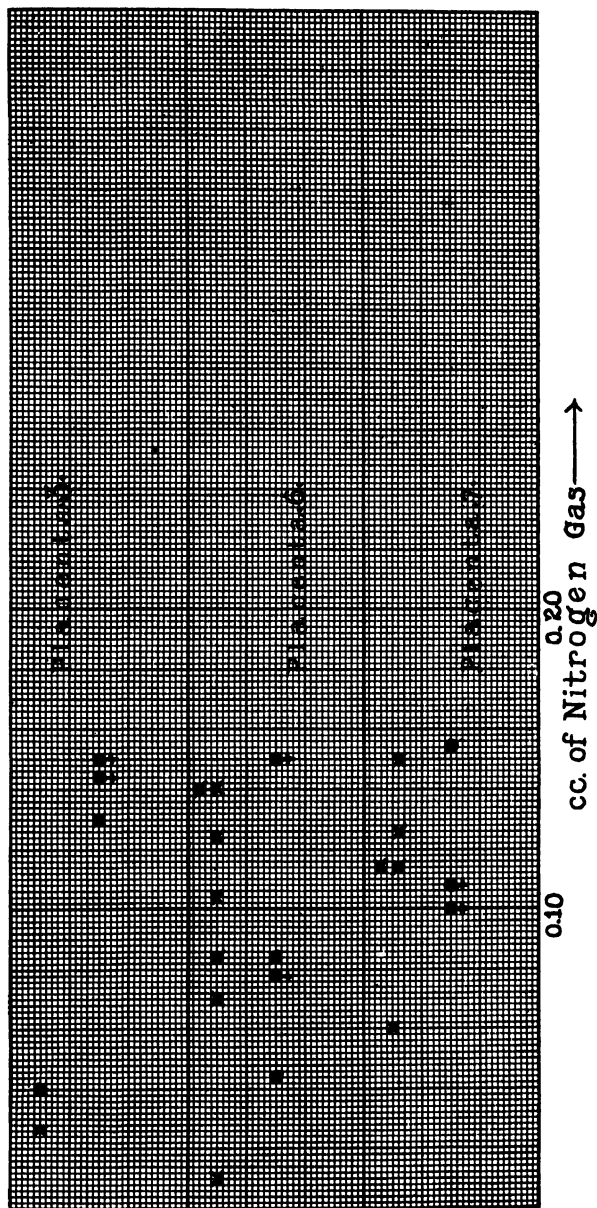


FIG. 3. (For explanation of symbols see p. 470.) Results from placentas prepared according to Abderhalden, except for initial washing with citrate, and preserved in dry powder form.



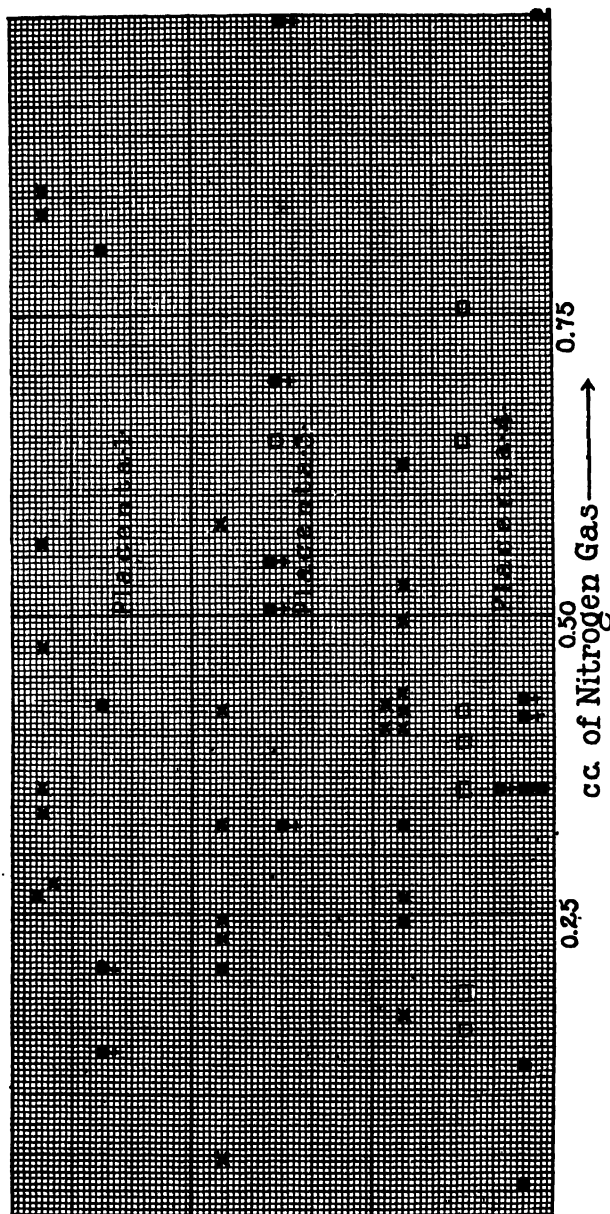


FIG. 4 (For explanation of symbols see p. 470.) Results from same class of placentas as in Fig. 3, but filtrates here were hydrolyzed before the amino nitrogen was determined. Comparison with Fig. 3 shows that hydrolysis about triples the increase in amino nitrogen in the serum filtrates, indicating that the average complexity of the "peptone" substances formed by the interaction of serum and placenta is about that of a tripeptide. Results are similar to those obtained without hydrolysis (other Figs.) in failing to show a marked or constant difference between pregnant and normal sera.

TABLE I A.

*Normal Men. Standard Method (Filtrates Not Hydrolyzed).*

No.	Condition.	Amount of serum.	Substrate.	Nitrogen gas obtained.	Increase observed.	Increase per 2 cc. of serum.
1	Normal	cc.		cc.	cc.	cc.
		2	—	0.25	—	—
		"	—	0.245	—	—
		"	—	0.255	—	—
		"	Placenta 6	0.33	0.08	0.08
2	Normal	2	—	0.275	—	—
		"	Placenta 6	0.39	0.115	0.115
		"	" 7	0.43	0.155	0.155
3	Normal	2	—	0.245	—	—
		"	—	0.255	—	—
		"	Placenta 6	0.32	0.07	0.07
		"	" 7	0.40	0.15	0.15
4	Normal	2	—	0.265	—	—
		"	Placenta 6	0.31	0.045	0.045
5	Normal	2	—	0.19	—	—
		"	—	0.185	—	—
		"	Placenta 6	0.27	0.083	0.083
		"	" 7	0.34	0.153	0.153
6	Normal	2	—	0.22	—	—
		"	Placenta 3	0.35	0.13	0.13
7	Normal	2	—	0.25	—	—
		"	—	0.245	—	—
		"	—	0.255	—	—
		"	Placenta 11	0.30	0.05	0.05
		"	" 12	0.31	0.06	0.06
		"	" 13	0.29	0.04	0.04
		"	" 14	0.32	0.07	0.07
		"	" 15	0.30	0.05	0.05
8	Normal	"	" 17	0.35	0.10	0.10
		2	—	0.275	—	—
		"	Placenta 11	0.28	0.005	0.005
		"	" 12	0.27	—	—
		"	" 13	0.27	—	—
		"	" 14	0.39	0.115	0.115
		"	" 15	0.45	0.175	0.175
		"	" 16	0.33	0.055	0.055
		"	" 17	0.40	0.125	0.125

TABLE I A.—*Concluded.*

No.	Condition.	Amount of serum.	Substrate.	Nitrogen gas obtained.	Increase observed.	Increase per 2 cc. of serum.
9	Normal	cc.	—	cc.	cc.	cc.
		2	—	0.28	—	—
		"	—	0.275	—	—
		"	Placenta 11	0.32	0.043	0.043
		"	" 12	0.30	0.023	0.023
		"	" 13	0.29	0.013	0.013
		"	" 14	0.33	0.053	0.053
		"	" 15	0.38	0.103	0.103
		"	" 16	0.33	0.053	0.053
		"	" 17	0.42	0.143	0.143
		"	Carcinoma uteri	0.38	0.103	0.103
10	Normal	2	—	0.245	—	—
		"	—	0.255	—	—
		"	Placenta 11	0.27	0.02	0.02
		"	" 12	0.275	0.025	0.025
		"	" 13	0.28	0.03	0.03
		"	" 14	0.33	0.08	0.08
		"	" 15	0.36	0.11	0.11
		"	" 16	0.34	0.09	0.09
		"	" 17	0.43	0.18	0.18
		"	Carcinoma uteri	0.46	0.21	0.21
11	Normal	2	—	0.265	—	—
		"	—	0.265	—	—
		"	Placenta 11	0.265	—	—
		"	" 12	0.260	—	—
		"	" 13	0.260	—	—
		"	" 14	0.30	0.035	0.035
		"	" 15	0.32	0.055	0.055
		"	" 16	0.27	0.005	0.005
		"	" 17	0.37	0.105	0.105
		"	Carcinoma uteri	0.32	0.055	0.055
12	Normal	2	—	0.255	—	—
		"	—	0.260	—	—
		"	Placenta 14	0.355	0.098	0.098
		"	" 15	0.35	0.093	0.093
		"	" 16	0.31	0.053	0.053
13	Normal	"	" 17	0.39	0.133	0.133
		2	—	0.19	—	—
		"	—	0.185	—	—
		"	Placenta 13	0.21	0.023	0.023
		"	" 14	0.31	0.123	0.123
		"	" 15	0.30	0.113	0.113
		"	" 16	0.31	0.123	0.123
		"	" 17	0.39	0.203	0.203
		"	Carcinoma uteri	0.408	0.221	0.221

TABLE I B.  
Non-Pregnant Women. Standard Method.

No.	Condition.	Amount of serum.	Substrate.	Nitrogen gas obtained.	Increase observed.	Increase per 2 cc. of serum.
14	Non-pregnant; breast abscess. Normal temperature Nov. 18. Blood was taken Nov. 24	cc. 2 "	— Placenta 3	cc. 0.38 0.60	cc. — 0.22	cc. — 0.22
15	Uterus prolapse; positive Wassermann	2 "	— Placenta 3	0.44 0.59	— 0.15	— 0.15
16	Normal	2 " "	— — Placenta 3	0.11 0.10 0.16	— — 0.055	— — 0.055
17	Normal	2 "	— Placenta 3	0.11 0.21	— 0.10	— 0.10
18	Normal	2 " " "	— Placenta 3 " 6 " 7	0.28 0.43 0.43 0.38	— 0.15 0.15 0.10	— 0.15 0.15 0.10
19	Normal	2 "	— Placenta 3	0.255 0.40	— 0.145	— 0.145
20	Non-pregnant; operated 7 days ago. Temperature normal	2 " " "	— — Placenta 6 " 7	0.21 0.195 0.28 0.31	— — 0.078 0.108	— — 0.078 0.108
21	Miscarriage at 4 mos., 6 mos. ago. Blood taken 6 days after operation. Temperature normal	1.5 " " " "	— Placenta 14 " 15 " 16 " 17	0.19 0.30 0.27 0.21 0.35	— 0.11 0.08 0.02 0.16	— 0.147 0.107 0.027 0.213
22	Normal	2 " " " " " "	— — Placenta 11 " 13 " 14 " 15 " 16 " 17	0.20 0.19 0.27 0.22 0.29 0.28 0.28 0.38	— — 0.075 0.025 0.095 0.085 0.085 0.185	— — 0.075 0.025 0.095 0.085 0.085 0.185

TABLE I B.—*Concluded.*

No.	Condition.	Amount of serum.	Substrate.	Nitrogen gas obtained.	Increase observed.	Increase per a cc. of serum.
23	Non-pregnant; delivered 5 mos. ago. Operated 7 days ago. Temperature normal	cc.	—	cc.	cc.	cc.
		"	—	0.21	—	—
		"	—	0.195	—	—
		"	Placenta 13	0.21	0.008	0.008
		"	" 14	0.28	0.078	0.078
		"	" 15	0.31	0.108	0.108
		"	" 16	0.25	0.048	0.048
		"	" 17	0.30	0.098	0.098
24	Normal	"	Carcinoma uteri	0.30	0.098	0.098
		1.5	—	0.16	—	—
		"	—	0.155	—	—
		"	Placenta 14	0.28	0.123	0.163
		"	" 15	0.23	0.073	0.100
		"	" 17	0.26	0.103	0.140
25	Normal	"	Carcinoma uteri	0.29	0.133	0.180
		2	—	0.20	—	—
		"	—	0.20	—	—
		"	Placenta 13	0.23	0.03	0.03
		"	" 14	0.32	0.12	0.12
		"	" 15	0.33	0.13	0.13
		"	" 16	0.26	0.06	0.06
		"	" 17	0.34	0.14	0.14
26	Never has been pregnant; normal	"	Carcinoma uteri	0.39	0.19	0.19
		2	—	0.23	—	—
		"	—	0.23	—	—
		"	Placenta 14	0.37	0.14	0.14
		"	" 15	0.41	0.18	0.18
		"	" 16	0.33	0.10	0.10
27	Normal	"	" 17	0.48	0.25	0.25
		2	—	0.28	—	—
		"	—	0.28	—	—
		"	Placenta 14	0.39	0.11	0.11
		"	" 15	0.43	0.15	0.15
		"	" 16	0.36	0.08	0.08
		"	" 17	0.46	0.18	0.18
		"	Carcinoma uteri	0.46	0.18	0.18
28	Normal	2	—	0.26	—	—
		"	—	0.25	—	—
		"	Placenta 14	0.33	0.075	0.075
		"	" 15	0.36	0.105	0.105
		"	" 16	0.33	0.075	0.075

TABLE I C.  
Pregnant Women. Standard Method.

No.	Condition.	Amount of serum.	Substrate.	Nitrogen gas obtained.	Increase observed.	Increase per 2 cc. of serum.
29	Pregnant 9 mos.	cc.		cc.	cc.	cc.
		2	—	0.26	—	—
		"	—	0.26	—	—
		"	Placenta 3	0.30	0.04	0.04
30	Pregnant 9 mos.	2	—	0.24	—	—
		"	—	0.23	—	—
		"	Placenta 3	0.35	0.115	0.115
		"	—	0.24	—	—
31	Pregnant 9 mos.	2	—	0.41	—	—
		"	Placenta 6	0.17	0.17	0.17
32	Incomplete abortion	2	—	0.34	—	—
		"	—	0.34	—	—
		"	Placenta 7	0.50	0.16	0.16
		"	—	0.20	—	—
33	Pregnant 5½ mos.	2	—	0.21	—	—
		"	—	0.29	0.085	0.085
		"	Placenta 6	0.32	0.115	0.115
		"	" 7	0.32	0.115	0.115
34	Pregnant 5 mos.	2	—	0.23	—	—
		"	—	0.22	—	—
		"	Placenta 6	0.33	0.105	0.105
		"	" 7	0.34	0.115	0.115
35	Pregnant 6 mos.	2	—	0.17	—	—
		"	—	0.18	—	—
		"	Placenta 6	0.30	0.125	0.125
		"	" 7	0.30	0.125	0.125
36	Pregnant 6 mos.	2	—	0.18	—	—
		"	Placenta 6	0.32	0.14	0.14
37	Pregnant 9 mos.; eclamptic case	2	—	0.28	—	—
		"	—	0.28	—	—
		"	Placenta 6	0.42	0.14	0.14
		"	—	0.23	—	—
38	Preeclamptic case	2	—	0.38	—	—
		"	Placenta 7	0.15	0.15	0.15
39	Pregnant 9 mos.	2	—	0.20	—	—
		"	Placenta 11	0.26	0.06	0.06
		"	" 13	0.265	0.065	0.065
		"	" 14	0.32	0.12	0.12
		"	" 15	0.36	0.16	0.16
		"	" 16	0.25	0.05	0.05
		"	" 17	0.44	0.24	0.24
		"	—	0.20	—	—

TABLE I C.—*Continued.*

No.	Condition.	Amount of serum.	Substrate.	Nitrogen gas obtained.	Increase observed.	Increase per a cc. of serum.
40	Pregnant 9 mos.	cc.	—	cc.	cc.	cc.
		2	—	0.19	—	—
		"	—	0.18	—	—
		"	Placenta 11	0.24	0.055	0.055
		"	" 13	0.21	0.025	0.025
		"	" 14	0.27	0.085	0.085
		"	" 15	0.30	0.115	0.115
41	Pregnant 9 mos.	"	" 16	0.24	0.055	0.055
		"	" 17	0.32	0.135	0.135
		2	—	0.20	—	—
		"	—	0.19	—	—
		"	Placenta 11	0.24	0.045	0.045
		"	" 13	0.20	0.005	0.005
		"	" 15	0.32	0.125	0.125
42	Pregnant 9 mos.	"	" 16	0.21	0.015	0.015
		"	" 17	0.36	0.165	0.165
		2	—	0.23	—	—
		"	—	0.235	—	—
		"	Placenta 11	0.290	0.068	0.068
		"	" 13	0.225	—	—
		"	" 14	0.355	0.123	0.123
43	Pregnant 9 mos.	"	" 15	0.350	0.118	0.118
		"	" 16	0.330	0.098	0.098
		"	" 17	0.360	0.128	0.128
		2	—	0.29	—	—
		"	—	0.285	—	—
		"	Placenta 11	0.37	0.082	0.082
		"	" 13	0.37	0.082	0.082
44	Pregnant 9 mos.	"	" 14	0.45	0.162	0.162
		"	" 15	0.53	0.242	0.242
		"	" 16	0.41	0.122	0.122
		"	" 17	0.64	0.352	0.352
		2	—	0.20	—	—
		"	—	0.20	—	—
		"	Placenta 11	0.33	0.13	0.13
45	Pregnant 9 mos.	"	" 13	0.23	0.03	0.03
		"	" 14	0.37	0.17	0.17
		"	" 15	0.40	0.20	0.20
		"	" 16	0.305	0.105	0.105
		"	" 17	0.45	0.25	0.25
		2	—	0.18	—	—
		"	Placenta 11	0.29	0.11	0.11
		"	" 13	0.20	0.02	0.02
		"	" 14	0.32	0.14	0.14
		"	" 15	0.29	0.11	0.11
		"	" 16	0.22	0.04	0.04

TABLE I C.—Continued.

No.	Condition.	Amount of serum.	Substrate.	Nitrogen gas obtained.	Increase observed.	Increase per cc. of serum.
		cc.		cc.	cc.	cc.
46	Pregnant 9 mos.	2	—	0.18	—	—
		"	—	0.185	—	—
		"	Placenta 13	0.18	—	—
		"	" 14	0.32	0.138	0.138
		"	" 15	0.35	0.168	0.168
		"	" 16	0.275	0.093	0.093
		"	" 17	0.42	0.238	0.238
47	Pregnant 5½ mos.	2	—	0.20	—	—
		"	—	0.21	—	—
		"	Placenta 13	0.22	0.015	0.015
		"	" 14	0.32	0.115	0.115
		"	" 15	0.31	0.105	0.105
		"	" 16	0.28	0.075	0.075
		"	" 17	0.395	0.190	0.190
		"	Carcinoma uteri	0.34	0.135	0.135
48	Pregnant 6 mos. (Examined)	2	—	0.20	—	—
		"	—	0.21	—	—
		"	Placenta 13	0.25	0.045	0.045
		"	" 14	0.31	0.105	0.105
		"	" 15	0.32	0.115	0.115
		"	" 16	0.31	0.105	0.105
		"	" 17	0.43	0.225	0.225
		"	Carcinoma uteri	0.36	0.155	0.155
49	Pregnant 5 mos.	2	—	0.23	—	—
		"	—	0.22	—	—
		"	Placenta 13	0.23	0.005	0.005
		"	" 15	0.39	0.165	0.165
		"	" 16	0.29	0.065	0.065
		"	" 17	0.41	0.185	0.185
50	Pregnant 6 mos.	2	—	0.17	—	—
		"	—	0.18	—	—
		"	Placenta 13	0.25	0.075	0.075
		"	" 15	0.28	0.105	0.105
		"	" 16	0.36	0.185	0.185
		"	" 17	0.28	0.105	0.105
51	Pregnant 6 mos.	2	—	0.18	—	—
		"	Placenta 14	0.38	0.20	0.20
		"	" 15	0.36	0.18	0.18
		"	" 16	0.28	0.10	0.10
		"	" 17	0.43	0.25	0.25
		"	Carcinoma uteri	0.38	0.20	0.20
52	Pregnant 6½ mos.	2	—	0.20	—	—
		"	—	0.20	—	—
		"	Placenta 14	0.43	0.23	0.23
		1	" 15	0.18	—	0.16
		2	" 16	0.31	0.11	0.11
		"	" 17	0.36	0.16	0.16
		"	Carcinoma uteri	0.38	0.18	0.18



TABLE I C.—*Concluded.*

No.	Condition.	Amount of serum.	Substrate.	Nitrogen gas obtained.	Increase observed.	Increase per 2 cc. of serum.
53	Eclamptic case, 9 mos. 6 convulsions	cc.	—	cc.	cc.	cc.
		2	—	0.28	—	—
		"	—	0.28	—	—
		"	Placenta 14	0.41	0.13	0.13
		"	" 15	0.42	0.14	0.14
		"	" 16	0.37	0.09	0.09
		"	" 17	0.46	0.18	0.18
54	Pregnant 9 mos.	"	Carcinoma uteri	0.43	0.15	0.15
		2	—	0.21	—	—
		"	Placenta 11	0.26	0.05	0.05
		"	" 12	0.24	0.03	0.03
		"	" 13	0.22	0.01	0.01
		"	" 15	0.38	0.17	0.17
		"	" 16	0.29	0.08	0.08
55	Preeclamptic case; pregnant 9 mos.	"	" 17	0.46	0.25	0.25
		2	—	0.22	—	—
		"	—	0.24	—	—
		"	Placenta 14	0.35	0.12	0.12
		"	" 15	0.36	0.13	0.13
		"	" 16	0.30	0.07	0.07
		"	" 17	0.45	0.22	0.22

TABLE II A.

*Men and Non-Pregnant Women. Filtrates Hydrolyzed.*

No.	Condition.	Amount of serum.	Substrate.	Nitrogen gas obtained.	Increase observed.	Increase per 2 cc. of serum.
56	Woman, uterus prolapse; positive Wassermann	cc.	—	cc.	cc.	cc.
		2	—	0.44	—	—
		"	—	0.46	—	—
		"	Placenta 3	1.00	0.55	0.55
57	Woman, non-pregnant; normal	"	" 5	1.68	1.23	1.23
		2	—	0.50	—	—
		"	Placenta 3	1.20	0.70	0.70
		"	" 5	0.91	0.41	0.41
58	Woman, non-pregnant; normal	2	—	0.35	—	—
		"	Placenta 1	0.55	0.20	0.20
		"	" 3	0.67	0.32	0.32
		"	" 5	0.70	0.35	0.35
59	Man, normal	1.4	—	0.29	—	—
		"	—	0.30	—	—
		"	Placenta 5	0.39	0.095	0.13
60	Man, normal	1.2	—	0.36	—	—
		"	Placenta 5	0.38	0.02	0.03

TABLE II A.—*Concluded.*

No.	Condition.	Amount of serum.	Substrate.	Nitrogen gas obtained.	Increase observed.	Increase per 2 cc. of serum.
61	Man, normal	cc.	—	cc.	cc.	cc.
		1	Placenta 1	0.225	—	—
		"	" 5	0.440 0.350	0.215 0.125	0.43 0.25
62	Woman, normal	1	—	0.177	—	—
		"	Placenta 3	0.690	0.513	1.03
63	Woman, normal	1	—	0.29	—	—
		"	Placenta 1	0.36	0.07	0.14
		"	" 3	0.56	0.27	0.54
		"	" 5	0.50	0.21	0.42

TABLE II B.

*Pregnant Women. Filtrates Hydrolyzed.*

No.	Condition.	Amount of serum.	Substrate.	Nitrogen gas obtained.	Increase observed.	Increase per 2 cc. of serum.
64	Pregnant 9 mos.	cc.	—	cc.	cc.	cc.
		2	Placenta 5	0.36 0.80	— 0.44	— 0.44
65	Pregnant 9 mos.	2	—	0.28	—	—
		"	Placenta 1	0.62	0.34	0.34
		"	" 3	0.515	0.235	0.235
66	Pregnant 9 mos.	2	—	0.26	—	—
		"	—	0.27	—	—
		"	Placenta 1	0.83	0.565	0.565
		"	" 3	0.68	0.415	0.415
67	Pregnant 9 mos.	2	—	0.26	—	—
		"	—	0.26	—	—
		"	Placenta 3	0.30	0.04	0.04
		"	" 5	0.44	0.18	0.18
68	Pregnant 9 mos.	2	—	0.26	—	—
		"	Placenta 5	0.50	0.240	0.240
69	Pregnant 9 mos.	2	—	0.25	—	—
		"	Placenta 1	0.60	0.35	0.35
70	Pregnant 9 mos.	2	—	0.20	—	—
		"	Placenta 3	0.44	0.24	0.24
		"	" 5	0.46	0.26	0.26

TABLE II B.—*Concluded.*

No.	Condition.	Amount of serum.	Substrate.	Nitrogen gas obtained.	Increase observed.	Increase per 2 cc. of serum.
71	Pregnant 9 mos.	cc.	—	cc.	cc.	cc.
		2	—	0.44	—	—
		"	—	0.42	—	—
72	Pregnant 9 mos.	"	Placenta 5	0.59	0.16	0.16
		2	—	0.34	—	—
		"	Placenta 1	1.18	0.84	0.84
73	Pregnant 9 mos.	2	—	0.41	—	—
		"	—	0.41	—	—
		"	Placenta 5	0.82	0.41	0.41
74	Pregnant 9 mos.	2	—	0.24	—	—
		"	—	0.24	—	—
		"	Placenta 1	0.72	0.48	0.48
		"	" 3	0.56	0.32	0.32
		"	" 5	0.76	0.52	0.52
75	Pregnant 9 mos.	2	—	0.38	—	—
		"	—	0.42	—	—
		"	Placenta 1	0.68	0.28	0.28
76	Pregnant 9 mos.	2	—	0.34	—	—
		"	Placenta 1	0.60	0.26	0.26
		"	" 5	0.66	0.32	0.32
77	Pregnant 9 mos.	2	—	0.42	—	—
		"	—	0.38	—	—
		"	Placenta 3	1.02	0.62	0.62
		"	" 5	1.02	0.62	0.62
78	Pregnant 9 mos.	2	—	0.38	—	—
		"	—	0.36	—	—
		"	Placenta 5	0.86	0.49	0.49
79	Incomplete abortion	2	—	0.44	—	—
		"	Placenta 5	0.84	0.40	0.40
80	Pregnant 9 mos.; specimen was in ice box 4 days before experiment was started	2	—	0.20	—	—
		"	Placenta 5	0.62	0.42	0.42

TABLE II C.  
*Pathological Cases. Filtrates Hydrolyzed.*

No.	Condition.	Amount of serum.	Substrate.	Nitrogen gas obtained.	Increase observed.	Increase per a cc. of serum.
81	Man; pneumonia	cc. 2 "	— Placenta 5	cc. 0.53 0.68	cc. — 0.15	cc. — 0.15
82	Man; pneumonia	2 "	— Placenta 5	0.50 0.68	— 0.18	— 0.18
83	Woman; pneumonia	2 "	— Placenta 5	0.37 0.76	— 0.39	— 0.39
84	Man; pneumonia; very sick	2 "	— Placenta 5	0.56 0.91	— 0.35	— 0.35
85	Man; pneumonia; temperature normal, convalescent	2 "	— Placenta 5	0.41 1.05	— 0.64	— 0.64
86	Woman; pneumonia	2 "	— Placenta 3	0.44 1.03	— 0.59	— 0.59
87	Man; pneumonia	2 "	— Placenta 5	0.57 1.32	— 0.75	— 0.75
88	Man; pneumonia	2 "	— Placenta 5	0.38 0.80	— 0.42	— 0.42

## NOTE ON THE MICRO-METHOD FOR GASOMETRIC DETERMINATION OF ALIPHATIC AMINO NITROGEN.<sup>1</sup>

BY DONALD D. VAN SLYKE.

(From the Hospital of The Rockefeller Institute for Medical Research.)

. (Received for publication, September 29, 1915.)

By slightly modifying the form of the gas burette the accuracy of the readings is so increased that the volume of the entire apparatus may be reduced to one-half that of the micro-amino apparatus described in our former paper (*i.e.*, so that only 1 cc. of solution is required for analysis), with corresponding reduction of the amount of material required to obtain results of the same percentage accuracy. The form of the burette is evident from the accompanying figure. The chief difference is that the zero point, instead of being placed at the bottom of the stopcock, is located on a capillary which extends for a few mm. below the cock. This permits marking off the upper boundary of the gas volume measured with an error of less than 0.001 cc. The burette, of 3 cc. capacity, is graduated into 0.01 cc. divisions about 1 mm. apart, so that by estimating tenths of a division gas volumes can be read to 0.001 cc. Such burettes must, of course, be accurately calibrated by the weight of water delivered.

A modification in the second stage of the determination (freeing the apparatus of air<sup>2</sup>) decidedly facilitates it. Instead of shak-

<sup>1</sup> The principle of the method and the original form of the apparatus were described in *Jour. Biol. Chem.*, 1911, ix, 185. The apparatus in its present form, but requiring tenfold the amount of material, was described in 1912, xii, 275. The application of the method to micro-analysis was described in 1913, xvi, 121. The present form of the apparatus, like those previously described, can be obtained from Emil Greiner, 55 Fulton St., New York.

<sup>2</sup> Van Slyke, D. D., *Jour. Biol. Chem.*, 1912, xii, 279.

ing back the nitrous acid mixture three times in the deaminizing chamber, one needs to shake only once, until sufficient nitric oxide gas has been formed to force the liquid in the chamber down to the mark indicating the amount of nitrous acid solution that should be in the chamber when the amino solution is added. One then closes cock *a*,<sup>3</sup> and so turns cock *c* that gases from the chamber can escape from the outlet tube at *c*. The deaminizing vessel is then shaken two minutes rapidly with the motor. The nitric oxide evolved

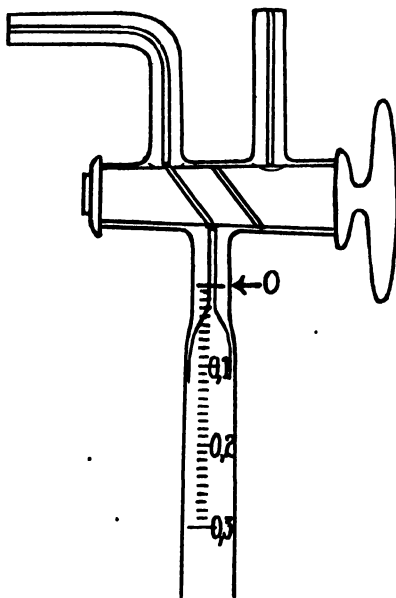


FIG. 1.

drives out the air as completely as it could be removed by the originally described mode of operation; in fact, with the micro-amino apparatus the removal seems to be slightly more complete, and the operation is considerably simpler. After the two minutes' shaking the deaminizing vessel is connected through *c* with the gas burette; and the determination finished as previously described.

It may be mentioned that the rubber connections, particularly that joining the deaminizing vessel to the gas burette, should be of

<sup>3</sup> See illustration, *Jour. Biol. Chem.*, 1912, xii, 278.

soft, heavy-walled rubber tubing. We use "stethoscope" tubing, which is soft and flexible, and has a wall 3 or 4 mm. thick.

The results in the following table were obtained with a 1 per cent solution of Kahlbaum's synthetic leucine.

	N <sub>2</sub> gas.	Tempera- ture.	Barometer.	Amino nitrogen.	
				Found.	Calculated.
	cc.	°C.	mm.	mg.	mg.
Solution measured in 1 cc. burette of apparatus. Burette correction = + 0.010 cc. Volume of solution analyzed = 1.01 cc. Weight of leucine = 10.10 mg.	1.957	25	757	1.081	1.080
	1.958	"	"	1.082	"
	1.957	"	"	1.081	"
	1.958	"	"	1.082	"
1.000 cc. of solution measured in calibrated Ostwald pipette and washed into deaminizing chamber	1.927	"	"	1.065	1.069
	1.932	"	"	1.068	"
	1.932	"	"	1.068	"

## A FURTHER STUDY ON THE BIOLOGIC CLASSIFICATION OF PNEUMOCOCCI.

By OSWALD T. AVERY, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

(Received for publication, September 17, 1915.)

The biologic classification of pneumococci, according to Dochez and Gillespie,<sup>1</sup> divides these organisms into four main groups. These groups represent well defined types which are distinct and readily differentiated by immunological reactions. In previous communications<sup>2</sup> the varieties of pneumococci, their relation to disease, and their significance in problems of specific therapy have been discussed in detail. The present work is confined to a study of a limited number of strains of pneumococci which, because of certain serological reactions, are closely allied to the second group, and appear to represent distinct subgroups of *Pneumococcus* Type II.

In classifying pneumococci by serological methods, agglutination and protection experiments have been employed. Agglutination of typical pneumococci of the second group in an immune serum of the homologous type is prompt and characteristic. There is occasionally encountered, however, an organism with which the agglutination reaction in Antipneumococcus Serum II is incomplete and less prompt, often being delayed several hours. The occurrence of strains showing these peculiarities has been frequent enough to direct attention to them, since in the presence of a positive, but atypical agglutination, some confusion may arise in diagnosis. Theoretically, too, it is of interest to determine, if possible, whether or not these variations in agglutinability indicate essential biologic differences.

The facts presented are a result of a study of ten strains of pneumococci, all of which agglutinated in Antipneumococcus Serum II, atypically as described above. Cross-immunity reactions and

<sup>1</sup> Dochez, A. R., and Gillespie, L. J., *Jour. Am. Med. Assn.*, 1913, lxi, 727.

<sup>2</sup> Cole, Rufus, *Arch. Int. Med.*, 1914, xiv, 56; *New York Med. Jour.*, 1915, ci, 1, 59. Dochez, A. R., and Avery, O. T., *Jour. Exper. Med.*, 1915, xxi, 114.



absorption tests indicate that these organisms possess characteristic group relationships among themselves and a common antigenic relation to the original and typical Type II pneumococcus.

*Description of Cultures.*—Ten strains of pneumococci were chosen, five of which were isolated from disease and five from the sputum of normal individuals. Table I gives the source and culture designation of the strains studied.

TABLE I.

Pneumococcus.	Source.	Obtained from	Remarks.
J	Acute maxillary antrum	Pus	Recovery.
L	" lobar pneumonia	Lung puncture	"
M	" " "	Blood	Septicemia, fatal.
Jn	" " "	Sputum	Recovery.
W	Primary pneumonia (child)	Lung puncture	Fatal.
As	Normal individual	Sputum	No infection.
Ar	" "	"	" "
F C B	" "	"	" "
S 13	Diabetic patient	"	" "
H	" "	"	" "

These ten strains possess the common characteristic of partial agglutination in antipneumococcus serum of Type II. Culturally and biologically they present all the usual characters of typical pneumococci: such as inulin fermentation, bile solubility, and more or less distinct capsule formation. Their virulence on isolation was distinctly lower than that of the typical Type II organism.

Agglutination as illustrated in Table II establishes a definite relationship between the strains studied and the type organism of Pneumococcus Group II. The reaction of all ten strains in Serum II is distinct, but never as prompt or complete as in the case of the typical Group II organism. With all typical Type II pneumococci the agglutination begins almost immediately and is complete in half an hour, while the reaction of these ten strains is always delayed and often incomplete at the end of the period of observation. It is further evident that these minor agglutinins tend to disappear in the higher dilution and are completely absent in dilution of 1 to 80, at which titer the reaction of the type organism still persists.

This variation in agglutination was at first attributed to the possibility that there might exist, among pneumococci of Group II,

## EXPERIMENTAL.

*Agglutination Experiments.*

TABLE II.

*Determination of the Titer of Agglutination of Strains in Antipneumococcus Serum II, Using Antipneumococcus Serum I and Normal Horse Serum (N) as Controls.*

Dilution.	1:1			1:10			1:20			1:40			1:80		
Sera.	N	I	II	N	I	II	N	I	II	N	I	II	N	I	II
Culture															
As 11 <sup>5</sup>	-	-	++	-	-	++	-	-	++	-	-	-	-	-	-
L 6 <sup>5</sup>	-	-	++	-	-	++	-	-	++	-	-	+	-	-	-
Jn 8 <sup>9</sup>	-	±	++	-	-	++	-	-	++	-	-	++	-	-	-
M 5 <sup>10</sup>	-	-	++	-	-	++	-	-	++	-	-	+	-	-	-
Ar 9 <sup>5</sup>	-	±	++	-	-	++	-	-	++	-	-	+	-	-	-
J 7 <sup>5</sup>	-	-	++	-	-	++	-	-	++	-	-	+	-	-	-
W 5 <sup>9</sup>	-	-	++	-	-	++	-	-	++	-	-	±	-	-	-
F C B 9 <sup>5</sup>	-	+	++	-	±	+	-	-	-	-	-	-	-	-	-
S 13 12 <sup>5</sup>	-	+±	++	-	-	++	-	-	+	-	-	-	-	-	-
H 7 <sup>5</sup>	-	+	++	±	-	+	-	-	+	-	-	±	-	-	-
II 46 <sup>11</sup>	-	-	++	-	-	++	-	-	++	-	-	++	-	-	++
I 115 <sup>5</sup>	-	+	-	-	+	-	-	+	-	-	+	-	-	±	-

The reactions were read after 2 hrs. at 37° C. and over night on ice.

The numerals following the culture indicate the animal passage; the exponent indicates the number of generations removed from the last passage.

TABLE III.

*The Effect of Increased Virulence upon the Agglutinability of the Atypical Group II Pneumococcus in Antipneumococcus Serum II.*

Antipneumococcus Serum II.	Dilution.			
	1:10	1:20	1:40	1:80
Pneumococcus				
M 0 <sup>9</sup>	++	++	±	-
M 5 <sup>11</sup>	++	++	±	-
L 0 <sup>9</sup>	++	++	-	-
L 6 <sup>11</sup>	++	++	±	-
Jn 0 <sup>9</sup>	+	±	-	-
Jn 10 <sup>5</sup>	++	+	-	-
F C B 1 <sup>16</sup>	++	-	-	-
F C B 11 <sup>5</sup>	++	-	-	-
II 46 <sup>11</sup>	++	++	++	++

The numerals after the culture indicate number of mouse passages.

The exponent indicates the number of generations removed from the last passage.

strains of poor agglutinability, analogous to similar conditions among other bacterial groups. It was also thought that, since these organisms were of lower virulence than the type organism, this fact might bear some relation to their agglutinability. Agglutination tests were carried out with certain atypical strains, the virulence of which had been enhanced by repeated animal passage. The effect of the animal passage on the agglutination titer was then determined. Four strains were chosen, the virulence of which was raised by mouse passage until it had attained a point comparable to that of the typical Type II pneumococcus, with a minimum lethal dose of 0.000001 cc. of broth culture.

It is evident from Table III that enhancing the virulence of these strains did not affect their agglutinability. The variations in agglutinations between these strains and the type pneumococcus appear to be not merely differences in agglutinability, but suggest rather the possibility that actual differences in agglutinogenic properties may characterize these organisms. To determine this a univalent immune serum was prepared by immunization of rabbits to each of the ten strains.

*Agglutination by Immune Rabbit Sera of the Ten Strains of Atypical Group II Pneumococci and the Results Obtained by Cross-Agglutination.*

TABLE IV.

*Agglutination of the Ten Strains by Homologous Sera and the Effect of Such Sera on Stock Cultures of Pneumococcus of Type II.*

Immune rabbit sera.										
Sera.	As		Ar		S <sub>13</sub>		F C B		H	
Culture.	As	II	Ar	II	S <sub>13</sub>	II	F C B	II	H	II
I : I	++	—	++	—	++	—	++	—	++	—
I : 10	++	—	++	—	++	—	++	—	++	—
I : 20	++	—	+	—	++	—	++	—	++	—
I : 40	++	—	±	—	++	—	±	—	++	—

Sera.	L		J		Jn		W		M	
Culture.	L	II	J	II	Jn	II	W	II	M	II
I : I	++	—	++	—	++	—	++	—	++	—
I : 10	++	—	++	—	++	—	++	—	++	—
I : 20	++	—	++	—	++	—	++	—	++	—
I : 40	++	—	++	—	++	—	++	—	+	—

Table IV shows that the rabbit immune atypical sera each agglutinated strongly the homologous strain of pneumococcus, but that none of these sera had any effect on the type culture of Group II. This failure of the antisera of the ten strains to agglutinate the Type II pneumococcus shows differences in the antigenic properties of these organisms and is in striking contrast to the positive reactions of agglutination with these same strains by Antipneumococcus Serum II (Table II). This failure of reversibility of the agglutination reaction would seem, therefore, to be due to actual differences in the agglutinogenic groups of these various organisms.

TABLE V.  
*Cross-Agglutination.*

Immune sera.	Pneumococcus cultures.									
	As	S <sub>13</sub>	F C B	Ar	L	J	Jn	W	M	II
As	++	-	-	-	++	-	++	-	++	-
S <sub>13</sub>	-	++	-	-	-	-	-	-	-	-
F C B	-	-	++	-	-	-	-	-	-	-
Ar	-	-	-	++	-	+	-	+	-	-
L	++	-	-	-	++	-	++	-	++	-
J	-	-	-	++	-	++	-	++	-	-
Jn	++	-	-	-	++	-	++	-	++	-
W	-	-	-	++	-	+	-	++	-	-
M	+	-	-	-	+	-	+	-	+	-
II	-	-	-	-	-	-	-	-	-	++
Normal rabbit	-	-	-	-	-	-	-	-	-	-
II	+	++	+	+	++	+	++	++	++	++

Table V reveals the striking fact that in a series of organisms wholly contained within Group II certain separate relationships are demonstrable. An analysis of this table indicates clearly the existence of three distinct groups which appear to represent subdivisions of the main Group II, in the antiserum of which all members of each of these subdivisions are agglutinated. For purposes of convenience these subdivisions will be referred to as Subgroups II A, II B, and II X, and are shown more clearly in Table VI.

Subgroup II A consists of four strains, the immune reactions of which are specific within the group, being identical with those of all other strains of the group, but bearing no relation to those of Subgroups II B, or II X. Similarly, Subgroup II B consists of three other strains characterized by the possession of specific immunity

TABLE VI.  
*Subgroup II A.*

Immune sera.	Pneumococcus cultures.				
	As	L	Jn	M	II
As	++	++	++	++	-
L	++	++	++	++	-
Jn	++	++	++	++	-
M	++	+	+	+	-
II	+	++	++	++	++

*Subgroup II B.*

Immune sera.	Pneumococcus cultures.			
	Ar	J	W	II
Ar	++	+	+	-
J	++	++	++	-
W	++	+	++	-
II	+	+	++	++

*Subgroup II X.*

Immune sera.	Pneumococcus cultures.			
	F C P	S 13	H	II
F C B	++	-	-	-
S 13	-	++	-	-
H	-	-	++	-
II	+	++	++	++

reactions, identical for members of this subgroup alone. The remaining three strains have been placed in Subgroup II X which, like the larger Group IV of the original biologic classification, is peculiar in that it seems to consist of a heterogeneous series of independent strains which do not cross in their immunity reactions with members of the other two subgroups or with each other. All, however, possess the common character of atypical agglutination in Antipneumococcus Serum II. This subgroup, like its prototype Group IV, seems to be infinitely variable, and to be characterized by the absence of cross-immunity reactions and by lower virulence.

*Protection Experiments.*—The protection of animals against infection is generally conceded to be one of the most specific of immunological reactions and hence one of the most satisfactory methods of classification. White mice were given intraperitoneally graduated doses of pneumococci and at the same time a fixed quantity of immune serum. All animals except the virulence controls received 0.2

cc. of immune serum intraperitoneally. This quantity of Immune Serum II as a rule protects mice against 0.01 cc. of broth culture of the homologous organism which, given alone, kills mice regularly in doses of 0.000001 cc. All animals surviving for five days were considered effectively protected.

In experimental pneumococcal infection the specificity of the protective power of an immune serum is evident only when the culture employed is fully virulent. Of the ten strains of pneumococci it was found possible to raise the virulence of seven by animal passage. The virulence of the other three strains could not be increased sufficiently for use in protection experiments, although they were passed successively through 11, 14, and 18 animals, respectively. They were even then of such low virulence that it was impossible to kill mice with the moderate doses necessary for the successful carrying out of the test. Of these three strains, two belonged to Subgroup II X and one to Subgroup II A.

*Further Evidence of the Specificity of Group Relationships by Protection Experiments.*

TABLE VII.

*Protective Action of Antipneumococcus Serum II.*

*The Relation between the Specificity of the Protective Action of Immune Serum and the Virulence of the Infecting Organism.*

*Pneumococcus L (Subgroup II A) after One Animal Passage.*

Pneumococcus L <sup>1</sup> .	Virulence controls.	Serum II.	Serum I.	Normal serum.
cc.				
0.01		S.*	D. 96	S.
0.001	D. 72	"	S.	"
0.0001	S.	"	"	D. 96
0.00001	"	"	"	S.
0.000001	D. 72	"	D. 96	D. 96

*Pneumococcus L (Subgroup II A) after Seven Animal Passages.*

Pneumococcus L <sup>1</sup> .	Virulence controls.	Serum II.	Serum I.
cc.			
0.01		S.	D. 18
0.001		"	" "
0.0001	D. 36	"	" 24
0.00001	" "	"	" 36
0.000001	" "	"	" "

\* In the tables D. stands for died; S. for survived. The figures represent the number of hours before the death of the animal.

Table VII shows that after increasing the virulence of pneumococcus L (Subgroup II A) to a degree sufficient to apply the test, definite protection was afforded by stock Antipneumococcus Serum II against 10,000 times the minimal lethal dose of culture. The odd survivals and non-specific reactions with the same strain before its virulence was raised by animal passage is evidence of the futility of attempting specific reactions of a protective nature with avirulent organisms. That this phenomenon does not represent a reversion to type brought about by animal passage is evidenced by the fact that the antigenic properties of these organisms remained unaffected by such treatment. Of the seven strains, the virulence of which was increased by mouse passage, Antipneumococcus Serum Type II protected against six, three of which belonged to Subgroup II A and three to Subgroup II B. The seventh strain, an organism of Subgroup II X, although made equally virulent by nine mouse passages, was not protected against by immune serum of Type II.

*Cross-Protection Tests with Homologous and Heterologous Sera of the Three Subgroups of Pneumococcus II.*

TABLE VIII.

*Protective Action of Sera of Subgroup II A against a Pneumococcus of the Same Group, and Failure of Sera of Subgroups II B and II X to Protect against the Same Organism.*

Pneumococcus Subgroup II A.	Immune sera.					Virulence controls.
	Subgroup II A.		Subgroup II B.	Subgroup II X.	Antipneumo- coccus Type II.	
Jn 9 <sup>1</sup>	Jn	L	W	F C B		
cc.	D. 72	S.	D. 18	D. 18	D. 96	
0.01	S.	"	" "	" "	S.	
0.001	"	"	" "	" "	"	D. 18
0.0001	"	"	" "	" "	"	" "
0.00001	"	"	" "	" 24	"	" "
0.000001	"	"	" "	" "	"	" 24

Tables VIII and IX demonstrate that an immune serum of Subgroups II A and II B protects against any organism of the homologous subgroup, but fails to protect against any strain of the other two subgroups. Table X emphasizes the individual character of organ-

TABLE IX.

*Protective Action of Sera of Subgroup II B against a Pneumococcus of the Same Subgroup and Failure of Sera of Subgroups II A and II X to Protect against the Same Organism.*

Pneumococcus Subgroup II B.	Immune sera.					Virulence controls.
	Subgroup II B.		Subgroup II A.	Subgroup II X.	Antipneumo- coccus Type II.	
W 61	W	J	Jn	F C B		
cc.	D. 26	S.	D. 18	D. 18	D. 96	
0.01	S.	"	" "	" "	S.	
0.001	"	"	" "	" "	"	D. 18
0.0001	"	"	" "	" "	"	" "
0.00001	"	"	" "	" "	"	" "
0.000001	"	"	" "	" "	"	" "

TABLE X.

*Protective Action of Serum of Subgroup II X against the Homologous Organisms Only and Failure of Sera of Subgroups II A and II B and Anti-pneumococcus Serum II To Protect against the Same.*

Pneumococcus Subgroup II X.	Immune sera.					Virulence controls.
	Subgroup II X.		Subgroup II A.	Subgroup II B.	Antipneumo- coccus Type II.	
F C B 111	F C B	S 13	Jn	W		
cc.	D. 18	D. 18	D. 18	D. 18	D. 18	
0.01	S.	" "	" "	" "	" "	
0.001	"	" "	" "	" "	" "	D. 18
0.0001	"	" "	" "	" "	" "	" "
0.00001	"	" "	" 22	" "	" "	" "
0.000001	"	" "	" "	" 36	" "	" "

TABLE XI.

*Lack of Protective Power of Immune Sera of Subgroups II A, II B, and II X against Typical Pneumococcus II.*

Pneumococcus Type II.	Immune sera.			Virulence con- trols.
	Subgroup I A.	Subgroup II B.	Subgroup II X.	
II 461	Jn	W	F C B	
cc.	D. 18	D. 18	D. 18	
0.01	" "	" "	" "	
0.001	" "	" "	" "	D. 18
0.0001	" "	" "	" "	" "
0.00001	" "	" "	" "	" "
0.000001	" "	" "	" 26	" "



isms of Subgroup II X. A serum produced by immunization with any given strain of this type protects against that particular organism and against no other. As previously noted, there is a complete lack of crossing in the immunity reactions of the individual members of Subgroup II X. Table XI shows that in protective action, as in agglutination (Table IV), the immune sera of Subgroups II A, II B, and II X have no effect on the typical pneumococcus of Type II. That these immunologic reactions between the original Type II pneumococcus and organisms of the subgroups are not reversible seems to indicate degrees of difference in antigenic characters. While the serological specificity of the subgroups definitely separates one from the other, nevertheless their immune reactions with the antisera of the typical Type II organism indicate that they are all biologically related. The correlation of these subgroups is further proven by absorption tests.

*Absorption Experiments.*—The phenomenon of specific absorption of agglutinins from an immune serum by the homologous organism was first described by Castellani.<sup>3</sup> This investigator found that from a polyvalent serum produced by immunization with two microorganisms of different species, the agglutinins for either one could be removed by fractional absorption with the homologous strain, while in a serum thus exhausted, the antibodies for the second organism remained intact. It has been shown also that in a univalent serum against *Bacillus typhosus* not only are agglutinins present for that organism alone, but that in the same serum there also exist partial or minor agglutinins for bacilli which are biologically similar, and which fall within the same general group. Absorption of a typhoid immune serum by *Bacillus typhosus* removes not only the agglutinins for that organism, but completely exhausts the serum of its minor antibodies for the closely allied organisms. This reaction, however, is not reversible, for removal of the partial agglutinins by absorption with a member of the intermediary species leaves the antibodies for *Bacillus typhosus* practically undiminished. The significance of this phenomenon in bacterial classification is obvious, and its applicability to the present study is evident in the following protocols.

<sup>3</sup> Castellani, A., *Ztschr. f. Hyg. u. Infektionskrankh.*, 1902, xl, 1.

*Specificity of Absorption Reaction. Absorption of Antipneumococcus Serum I with Pneumococci of Groups I and II.*

*Technique.*—Specific absorption. Antipneumococcus Serum I. 2 cc. of serum were diluted with 3 cc. of salt solution. To the 5 cc. of diluted serum the live, washed bacterial residue of 150 cc. of a twenty-four hour broth culture of *Pneumococcus* I was added, allowed to stand in contact over night in the ice box, then centrifuged, the clear supernatant serum pipetted off, and passed through a Berkefeld filter.

Non-specific absorption. Antipneumococcus Serum I was absorbed with *Pneumococcus* II. The technique was the same as above.

Control. Antipneumococcus Serum I diluted, and, without the addition of any bacteria, filtered by the same technique.

TABLE XII.

*Specific and Non-Specific Absorption of Agglutinins from Antipneumococcus Serum I by Pneumococci of Groups I and II.*  
*Agglutination.*

Culture pneumococcus, Group I.	Antipneumococcus Serum I.		
	Specific absorption with <i>Pneumococcus</i> I.	Non-specific absorption with <i>Pneumococcus</i> II.	Control, Serum unabsorbed.
	—	++	++

TABLE XIII.

*Protective Power of Antipneumococcus Serum I after Absorption with Pneumococci of Groups I and II.*  
*Protection.*

Culture pneumococcus, Group I.	Virulence controls.	Antipneumococcus Serum I.		
		Specific absorption with <i>Pneumo-</i> <i>coccus</i> I.	Non-specific absorption with <i>Pneumococcus</i> II.	Control. Serum unabsorbed.
cc.				
0.01		D. 18	S.	S.
0.001		" 20	"	"
0.0001	D. 18	" 24	"	"
0.00001	" "	" "	"	"
0.000001	" 20	" 48	"	"

Tables XII and XIII demonstrate the specificity of the absorption reaction with pneumococci of the fixed Types I and II. Saturating an immune serum of Group I with pneumococcus of the same type completely exhausts that serum of all its agglutinins and protective antibodies, while absorption of the same serum with organisms of Group II does not appreciably diminish these immune substances for pneumococci of Group I.

*Absorption of Antipneumococcus Serum II with Pneumococcus II and Organisms of Its Subgroups II A, II B, and II X.*

**Technique.**—To 10 cc. of undiluted Antipneumococcus Serum II was added the washed bacterial residue from 150 cc. of an eighteen hour broth culture of the given strain of pneumococcus. Before being added to the serum the bacteria were killed by heating at 56° C. for forty-five minutes. The serum mixtures were incubated in the water bath for two hours at 37° C. and allowed to remain in contact over night in the ice box. The clumps of agglutinated bacteria were whirled out by centrifugation, and the clear supernatant serum was pipetted off. This serum was absorbed a second time by the same technique, tested for the absence of agglutinins, and called exhausted serum.

TABLE XIV.

*Cross-Agglutination Reactions with Antipneumococcus Serum II Exhausted by Absorption with Type Strains of Pneumococcus II and Its Subgroups II A, II B, and II X.*

Antipneumococcus Serum II. Absorbed by	Pneumococcus.									
	Subgroup II A.				Subgroup II B.			Subgroup II X.		
	Jn	As	I.	M	W	Ar	J	S 13	FCB	H
Subgroup A, Jn	—	—	—	—	++	±	++	++	+	++
" B, W	++	++	++	±	—	—	—	++	+	++
" X, S 13	++	++	++	±	++	++	++	—	+	++
Group II	—	—	—	—	—	—	—	—	—	—

Table XIV shows that specific absorption of Antipneumococcus Serum II with the typical Type II pneumococcus removes all the agglutinins, not only for the homologous organism, but also all the partial agglutinins for its subgroups, II A, II B, and II X. In other words, specific absorption of Antipneumococcus Serum II completely exhausts it of both major and minor agglutinins. Conversely, however, absorption of the same immune serum with a representative strain of Subgroup II A removes the minor agglutinins for members of that subgroup only, leaving intact the antibodies for the Type II pneumococcus and its other subgroups, II B and II X. Similarly, absorption of Antipneumococcus Serum II with any member of Subgroup II B takes out the agglutinins for all the Subgroup II B organisms, but leaves unaffected the antibodies for the Type II pneumococcus and its subgroups, II A and II X. The lack of cross-immunological reactions among the heterogeneous organisms within

Subgroup II X already noted in the previous agglutination and protective experiments (Tables VI and X) is again evident in the absorption tests. Saturation of Immune Serum II with a pneumococcus of Subgroup II X robs the serum of its agglutinins for that individual strain only, and for no other.

The results obtained by absorption experiments with Antipneumococcus Serum II, a serum produced by intensive immunization of the horse with a single strain of the typical Type II pneumococcus, corroborate the same specific groupings obtained by the cross-immunological reactions of agglutination and protection with immune rabbit sera of the individual strains.

#### DISCUSSION.

The biologic classification of the pneumococcus distinguishes four distinct groups. These types are based upon well defined immunologic differences. The accuracy with which these groups may be differentiated and the constancy of their relative frequency in disease and health emphasize the importance of their recognition in clinical and epidemiological studies. The exactness with which the large number of strains studied have conformed to type indicates the extraordinary uniformity and comparative fixity of the specific groups. These distinctive differences in antigenic properties not only offer a reliable method for the more exact determination of the varieties of pneumococcus, but afford the only rational basis for the study of immunotherapy in pneumococcal infection.

The second group of pneumococci of the original classification consists of highly virulent organisms which are responsible for about one-third of all cases of lobar pneumonia of pneumococcus origin. Organisms of this group produce infections which are clinically severe, and the mortality of which is about 35 per cent. The serological reactions by means of which Group II was originally identified are sharply defined. So characteristic and prompt is the agglutination of any strain of this type in Antipneumococcus Serum II that any deviation from the normal reaction is quickly recognized. The isolation of an occasional strain of pneumococcus which agglutinates atypically in Antipneumococcus Serum II led to an attempt to determine the nature of these organisms and their relation to the

type pneumococcus of Group II. Of the ten strains studied five were isolated from disease and five from the sputum of normal individuals. These organisms all exhibited the usual cultural and biochemical characteristics of the pneumococcus; namely, inulin fermentation, bile solubility, and varying degrees of capsular development. The facts developed by this study indicate the existence of pneumococci which are biologically similar and closely allied to the typical organism of Group II. These organisms possess partial antigenic characters common to the Type II pneumococcus, but they vary from the typical representative of this group by a diversity of relationships among themselves, and by a lack of the reversibility of their immune reactions with the type organism. Because of these variations these organisms may be classified as subvarieties of *Pneumococcus* Group II. All strains of the three subgroups thus far recognized are agglutinated by Antipneumococcus Serum II, but the diminished intensity of the reaction serves to distinguish them from typical II pneumococci. The incomplete reaction of agglutination of these subvarieties in the immune serum of Type II is apparently similar to the diminished reactions which occur in many immune sera with other organisms closely allied to the type used in producing the serum. Such reactions occur only in the higher concentrations of immune sera and may be attributed to the so called minor agglutinins. In such cases, also, a non-reversibility of the immune reaction has been noted. For instance, a potent typhoid immune serum may agglutinate *Bacillus coli* in the higher concentrations, but an anticolon serum may not affect *Bacillus typhosus*. All strains of these subgroups of pneumococci are partially agglutinated in the higher concentrations of Antipneumococcus Serum II, while conversely the immune serum produced by any strain of these subgroups fails to react with the typical II pneumococcus.

In addition to the partial agglutination of these strains in Antipneumococcus Serum II and the absence of reverse immunity reactions, these subvarieties are further characterized by certain interrelationships of a definite antigenic nature, by virtue of which they may be classified into at least three subgroups, which have been called Subgroups II A, II B, and II X. It has been shown in the preceding protocols that a given member of either Subgroup II A or

II B is characterized by the possession of immunity reactions identical with those of all other strains of the homologous subgroup, and that these reactions are specific only within the group. Subgroup II X is peculiar in that it seems to consist of a heterogeneous series of independent strains which do not cross in their immunity reactions with members of the other two subgroups or with each other. This subgroup, like its prototype, Group IV, of the original biologic classification, is of lower virulence, infinitely variable in its composition, and lacking in cross-immunity reactions. As has been noted, serum Type II fails to protect against organisms of this subgroup, and inasmuch as specific protection is regarded as the ultimate criterion for classification, it is doubtful whether organisms of Subgroup II X are of sufficiently close relationship to be included within Group II.

The specificity of these subgroups, II A and II B, as tested by the immunity reactions of agglutination and protection is further confirmed by the phenomenon of absorption. Saturation of Antipneumococcus Serum II with a typical Group II pneumococcus removes all the agglutinins both for the type organism and its three subgroups. Absorption of the same serum, however, with a member of either Subgroup II A or II B removes only the partial agglutinins for the homologous subgroup, but leaves intact the antibodies for the typical II pneumococcus and the other subgroup. Absorption of Antipneumococcus Serum II, on the other hand, by any member of Subgroup II X takes out the antibodies for that particular strain only, and for no other.

In the present discussion no attempt is made to interpret the experimental data in terms of their phylogenetic significance. Whether the subvarieties of the second group of pneumococci represent strains which have acquired independently certain adaptive characters, or whether they are related to each other and to the fixed type by the lineage of common descent is interesting. However, the limited nature of the present study precludes the formulation of any hypothesis as to origin.

## SUMMARY.

1. At least three subgroups of *Pneumococcus* Type II may be recognized by specific immune reactions. They have been called Subgroups II A, II B, and II X.

2. That the organisms of these three subgroups are biologically related to *Pneumococcus* Type II is shown by the following facts: (a) Agglutination with Antipneumococcus Serum II. (b) Protection with Antipneumococcus Serum II, except Subgroup II X. (c) Absorption of Antipneumococcus Serum II with typical Type II pneumococcus removes the antibodies for all subgroups. (d) Absorption of Antipneumococcus Serum II with a member of Subgroups II A or II B removes only the antibodies for the homologous subgroup. Absorption of Antipneumococcus Serum II with any given member of Subgroup II X removes the antibodies for that particular strain only.

3. That the three subgroups, although biologically related to *Pneumococcus* Type II, possess, nevertheless, specific differential characters which separate them one from another, is evidenced by the following facts: (a) The organisms of any subgroup are not agglutinated by the antisera of the other two subgroups. (b) They are not protected against by the sera of the other subgroups. (c) They do not absorb from Antipneumococcus Serum II the specific immune bodies of the other subgroups.

4. Subgroups II A and II B are characterized by immunity reactions identical within the respective group.

5. Subgroup II X consists of heterogeneous strains which do not cross in their immunity reaction with each other or with Subgroups II A or II B.

The author acknowledges his indebtedness to Dr. A. L. Bloomfield of the Johns Hopkins Hospital for five of the cultures of pneumococcus used in this study.

## A COMFORTABLE ARRANGEMENT FOR THE SEPARATE COLLECTION OF URINE AND FECES OF MALE INFANTS.\*

BY ANGELIA M. COURTNEY AND HELEN L. FALES.

(From the Laboratories of The Rockefeller Institute for Medical Research and the Babies' Hospital.)

Of the utmost importance in the quantitative investigation of infant excretions is the absolutely complete collection of the separate urine and feces for the desired period. Hardly second in importance is the question of comfort for the child, for it is possible that a constrained or unusual posture appreciably affects the metabolism. For example, one arrangement, in which the body is partly supported as in a hammock, brings considerable pressure on the lower part of the back and in some cases has seemed to cause increased peristalsis. With another method the bed is necessarily slightly tilted and the child held from slipping by an elaborate system of straps so arranged as not to press on any part of the body but holding shoulders and arms in an almost immovable position.

The arrangement here described is recommended as combining accuracy of collection with a maximum of comfort for the child. It is mainly a satisfactory combination of details from methods in use. The Finkelstein<sup>1</sup> rubber and glass contrivance, which experience in the Babies' Hospital has shown to be the most accurate for collecting urine, is somewhat modified to suit an entirely different arrangement for supporting the child. The means used to hold the child from slipping, although adopted independently here, is practically the same as that employed in Talbot's<sup>2</sup> metabolism bed. An

\*Thanks are due to Dr. E. A. Morgan, Resident Physician of the Babies' Hospital, for taking photographs.

1. Bendix and Finkelstein: Deutsche med. Wchnschr., 1900, xlii, 672.

2. Talbot, F. B.: Apparatus for Metabolism Experiments in Male Infants, Jour. Am. Med. Assn., Nov. 27, 1908, p. 1818.



air cushion is used in Schabad's<sup>8</sup> scheme, but so far as is known no use has elsewhere been made of the air cushion with an opening in the rim. This method can be carried out on any crib or bed by making the necessary modifications of the mattresses. It is more convenient to use an adjustable crib in which the bottom can be raised to bring the child to a convenient level for handling.

The following articles are required :

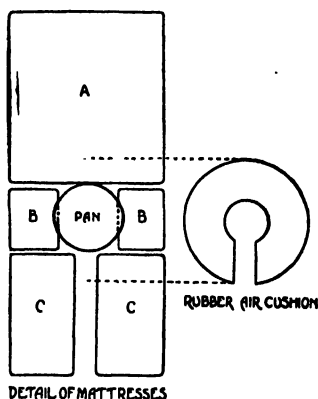
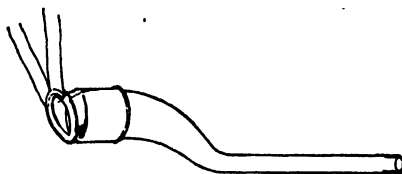


Fig. 1.—Diagram showing detail of mattress and rubber air cushion.

1. Mattresses, all of the ordinary thickness. One piece (*A*) 20 x 23 inches; two pieces (*B*) 6 x 8 inches; two pieces (*C*) 8 x 16 inches, for a crib 21 x 48 inches.
2. Rubber air cushion. Outside diameter 12 inches; inside diameter  $3\frac{1}{2}$  inches. Opening when inflated about 2 inches.



GLASS TUBE WITH FINKELSTEIN ATTACHMENT

Fig. 2.—Drawing of glass tube with Finkelstein attachment.

3. Cloth cover for cushion, consisting of two 18-inch squares with a circle  $3\frac{1}{2}$  inches in diameter cut out of the center, the pieces being stitched together around the circle and free on all sides.
3. Schabad : Arch. f. Kinderh., 1908, xlviii, 402.



Fig. 3.—Material of metabolism bed assembled.



Fig. 4.—Infant in the metabolism bed with apparatus applied.

4. Enamel ware pan with flange, 9 inches in diameter.
5. Ordinary male glass urinal.
6. Finkelstein rubber attachment.
7. Glass tube (after Finkelstein, but lighter and differently bent).
8. Cloth strap made of two pieces of webbing, each 6 inches wide, one 28 inches long, placed on the other which is 36 inches long, and the two stitched together across the middle.

Directions for setting up:

Cover mattresses separately with sheets, pinning on under side.

Place mattresses and pan in position according to diagram Fig. 1.

It is convenient to lay a circle of oiled silk in the bottom of the pan.

Inflate rubber cushion not too hard, place within cloth cover, and pin down snugly on mattresses, with opening toward bottom of bed.

Place cloth band across mattress A with lower edge just above lower edge of mattress and pin ends of bottom strap tightly to sides of mattress.

Place child on bed with anus directly over hole in rubber cushion, buttocks resting comfortably on cushion. If necessary, lay a pad under back above where he rests on the cushion.

Insert the large end of the glass tube (Fig. 2) in the end of the rubber attachment, pushing it in well.

Place small end of glass tube in neck of urinal and make the latter secure between cushions C.

Attach the rubber to the child, putting scrotum and penis within the large end of the glass tube and bring the rubber close to the body. Draw the rubber straps around the body, crossing on the back and fasten ends together in front with adhesive plaster. Make secure but not tight. Place absorbent cotton under rubber wherever it seems advisable.

Pin the ends of the upper strap of the cloth band snugly around the body.

The infant's legs rest on cushions C.

Ankles should be tied loosely by wide tapes, with absorbent cotton under the tape, to the sides of the crib, allowing some movement of the legs but not enough to cause disturbance of the urinal.

The glass tube in the rubber attachment resting in the space be-

tween the ends of the air cushion gives sufficient drop to assure the flow of the urine into the urinal.

Cushion B can easily be removed to facilitate changing the enamel pan.

A thin board between springs and mattresses will prevent any air currents from reaching the child from beneath.

With a very small or very restless child frequent inspection at the beginning is advisable to make sure that the rubber adjustment has not slipped, but ordinarily the necessary visits at intervals to remove the samples of urine and feces to the icebox are sufficient to make sure that all is right.

# INDEX TO VOLUME XXIV.

## **A**BDERHALDEN reaction, 458

Abderhalden serum test for cancer, results of applying a quantitative method to, 321

Acid,  $\alpha$ - $\alpha$ -anhydro-mucic and *d*-lyxohexosaminic, 134

Acid, electrolytes in the diffusion of, into the egg of *Fundulus*, 218

Acid, *d*-lyxohexosaminic and  $\alpha$ - $\alpha$ -anhydro-mucic, 134

Acids, amino-, improvements in the method for analysis of proteins by determination of the chemical groups characteristic of, 356

Acids, epimeric monocarboxylic sugar, relation between configuration and rotation of, 154

Acids, normal tridecylic and tetracosanic, synthesis of, 149

Adrenals, pathological action of arsenicals on, 93

Agglutination of bacteria *in vivo*, 67

Aliphatic amino nitrogen, micro-method for gasometric determination of, 486

ALLEN, C. H. See LEVENE, WEST, ALLEN, and VAN DER SCHEER, 149

ALLEN, FREDERICK M. Metabolic studies in diabetes, 325

——. Note concerning exercise in the treatment of severe diabetes, 455

——. Prolonged fasting in diabetes, 361

Amino-acids, improvements in method for analysis of proteins by determination of the chemical groups characteristic of, 356

Amino nitrogen, aliphatic, micro-method for gasometric determination of, 486

Analysis of proteins, improvements in method for, by determination of chemical groups characteristic of the different amino-acids, 356

$\alpha$ - $\alpha$ -Anhydro-mucic and *d*-lyxohexosaminic acid, 134

Antipneumococcus serum, mechanism of curative action of, 40

Arsenic, chemopathological studies with compounds of, 75, 83, 93

Arsenic kidney, types of, 75

Arsenic kidneys, histological changes in, 83

Arsenic therapy, specific, and lesions produced by arsenicals, 1

Arsenicals, lesions produced by, and their bearing on the problem of specific arsenic therapy, 1

Arsenicals, pathological action of, on the adrenals, 93

Arthritis, experimental, in rabbit. Contribution to pathogeny of arthritis in rheumatic fever, 109

AVERY, OSWALD T. A further study on the biologic classification of pneumococci, 489

**B**ACILLI, typhoid, injected intravenously into normal rabbits, fate of, 58

*Bacillus influenzae*, immunological study of, 28

Bacillus of Koch-Weeks, epidemic of conjunctivitis caused by, associated with cell inclusion conjunctivitis, bacteriological and clinical studies of, 13

Bacteria, agglutination of, *in vivo*, 67

Bactericidal action of ethylhydrocuprein on pneumococci, 421

Bacteriological and clinical studies of an epidemic of Koch-Weeks bacillus conjunctivitis associated with cell inclusion conjunctivitis, 13

Bacteriological results in chronic leukemia and pseudoleukemia, 123

Biologic classification of pneumococci, 489

Biology of peripheral nerves in transplantation, 156

BROWN, WADE H. Lesions produced by arsenicals and their bearing on the problem of specific arsenic therapy, 1

BROWN, WADE H., and PEARCE, LOUISE. Chemopathological studies with compounds of arsenic. III. On the patho-

- logical action of arsenicals on the adrenals, 93
- BROWN, WADE H. See PEARCE and BROWN, 75, 83
- BULL, CARROLL G. The mechanism of the curative action of antipneumococcus serum, 40
- A method of serum treatment of pneumococcal septicemia in rabbits, 49
- The fate of typhoid bacilli when injected intravenously into normal rabbits, 58
- The agglutination of bacteria *in vivo*, 67
- BUNKER, H. A., JR. See KARSNER, BUNKER, and GRABFIELD, 102
- BUNTING, C. H., and YATES, J. L. Bacteriological results in chronic leukemia and in pseudoleukemia, 123
- C**ALCIUM in permeability and irritability, 238
- Cancer, results of applying a quantitative method to the Abderhalden serum test for, 321
- CATTELL, McKEEN. See LOEB and CATTELL, 193
- Cell inclusion conjunctivitis associated with epidemic of Koch-Weeks bacillus conjunctivitis, bacteriological and clinical studies of, 13
- Cell, influence of electrolytes upon diffusion of potassium out of, and into, 193
- CHAMBERLAIN, MARY MITCHELL. See LOEB and CHAMBERLAIN, 229
- Chemical groups characteristic of different amino-acids, analysis of proteins by determination of, 356
- Chemical study of woman's milk, especially its inorganic constituents, 380
- Chemopathological studies with compounds of arsenic, 75, 83, 93
- Chemotherapy of experimental pneumococcal infection, 368
- Chlorides and urea in man, numerical laws governing rate of excretion of, 285
- Clinical and bacteriological studies of an epidemic of Koch-Weeks bacillus conjunctivitis associated with cell inclusion conjunctivitis, 13
- Clinical and electrocardiographic studies on the action of digitalis, 403
- COHEN, MARTIN. See NOGUCHI and COHEN, 13
- COHN, ALFRED E. Clinical and electrocardiographic studies on the action of digitalis, 403
- Conjunctivitis caused by Koch-Weeks bacillus, epidemic of, associated with cell inclusion conjunctivitis, bacteriological and clinical studies of, 13
- COURTNEY, ANGELIA M., and FALES, HELEN L. A comfortable arrangement for the separate collection of urine and feces of male infants, 505
- Studies in infant metabolism and nutrition, V. The composition and preparation of protein milk (*Eiweissmilch*), 308
- COURTNEY, ANGELIA M. See HOLT, COURTNEY, and FALES, 380
- D**ETERMINATION, gasometric, of aliphatic amino nitrogen, micro-method for, 486
- Determination of the chemical groups characteristic of the different amino-acids, improvements in method for analysis of proteins by, 356
- Diabetes, metabolic studies in, 325
- Diabetes, prolonged fasting in, 361
- Diabetes, severe, exercise in treatment of, 455
- Digitalis, clinical and electrocardiographic studies on the action of, 403
- Drugs and pathological conditions, influence of, on excretion, 285
- E**GG of *Fundulus*, rôle of electrolytes in diffusion of acid into, 218
- Eiweissmilch* (protein milk), composition and preparation of, 308
- Electrocardiograms, changes in, accompanying experimental changes in rabbits' hearts, 273
- Electrocardiographic and clinical studies on the action of digitalis, 403
- Electrolytes in the diffusion of acid into the egg of *Fundulus*, 218
- Electrolytes, influence of, upon diffusion of potassium out of the cell and into the cell, 193
- Epidemic of Koch-Weeks bacillus conjunctivitis associated with cell inclusion conjunctivitis, bacteriological and clinical studies of, 13
- Epimeric monocarboxylic sugar acids, relation between configuration and rotation of, 154
- Ethylhydrocuprein, bactericidal action of, on pneumococci, 421
- Ethylhydrocuprein (optochin), action of, *in vitro* and *in vivo*, on type strains of pneumococci, and on some other microorganisms *in vitro*, 250
- Excretion, influence of pathological conditions and of drugs on, 285

Excretion of urea and chlorides in man, numerical laws governing rate of, 285

Experimental arthritis in the rabbit. Contribution to the pathogeny of arthritis in rheumatic fever, 109

Experimental changes in rabbits' hearts, changes in electrocardiograms accompanying, 273

Experimental pneumococcal infection, chemosotherapy of, 368

FABER, HAROLD KNIEST. Experimental arthritis in the rabbit. A contribution to the pathogeny of arthritis in rheumatic fever, 109

FALES, HELEN L. See COURTNEY and FALES, 308, 505

— See HOLT, COURTNEY, and FALES, 380

Fasting, prolonged, in diabetes, 361

Feces and urine of male infants, a comfortable arrangement for separate collection of, 505

Fever, rheumatic, pathogeny of arthritis in, 109

FRASER, FRANCIS R. Changes in the electrocardiograms accompanying experimental changes in rabbits' hearts, 273

GASOMETRIC determination of aliphatic amino nitrogen, micro-method for, 486

Gladin, lactalbumin, and the protein of the rice kernel, some products of hydrolysis of, 335

Glucosone, action of aseptic tissue on, 139

GRABFIELD, G. P. See KARSNER, BUNKER, and GRABFIELD, 102

Growth of spontaneous tumors in mice, effect of Roentgen rays on rate of, 128

HEARTS of rabbits, changes in electrocardiograms accompanying experimental changes in, 273

Histological changes in arsenic kidneys, 83

HOLT, L. EMMETT, COURTNEY, ANGELIA M., and FALES, HELEN L. A chemical study of woman's milk, especially its inorganic constituents, 380

Hydrolysis of gliadin, lactalbumin, and the protein of the rice kernel, some products of, 335

Hydrolysis, partial, of proteins, kyrene fraction obtained on, 142

IMMUNOLOGICAL study of *Bacillus influenzae*, 28

*In vitro* and *in vivo* action of ethylhydrocuprein (optochin) on type strains of pneumococci, and on some other microorganisms *in vitro*, 250

*In vivo* agglutination of bacteria, 67

*In vivo* and *in vitro* action of ethylhydrocuprein (optochin) on type strains of pneumococci, and on some other microorganisms *in vitro*, 250

Infected animals with pneumonia and normal animals, action of lethal dose of strophanthin in, 438

Infection, experimental pneumococcal, chemosotherapy of, 368

*Influenza*, *Bacillus*, immunological study of, 28

INGEBRIGTSEN, RAGNVALD. A contribution to the biology of peripheral nerves in transplantation, 156

Injection, intravenous, of typhoid bacilli, into normal rabbits, 58

Inorganic constituents of woman's milk, chemical study of, 380

Insufflation, intrabronchial, of unorganized substances, production of pneumonic lesions by, 132

Intrabronchial insufflation of unorganized substances, production of pneumonic lesions by, 132

Intravenously injected typhoid bacilli into normal rabbits, fate of, 58

Irritability and permeability, calcium in, 238

Irritation, effect of, on permeability of meninges for salvarsan, 267

JAMIESON, ROSS A. The action of the lethal dose of strophanthin in normal animals and in animals infected with pneumonia, 438

KARSNER, H. T., BUNKER, H. A., JR., and GRABFIELD, G. P. A note on the immediate effects of reduction of kidney substance, 102

Kidney, arsenic, types of, 75

Kidney substance, immediate effects of reduction of, 102

Kidneys, arsenic, histological changes in, 83

KLINE, B. S., and MELTZER, S. J. Production of pneumonic lesions by intrabronchial insufflation of unorganized substances, 132

Koch-Weeks bacillus conjunctivitis, epidemic of, associated with cell inclusion conjunctivitis, bacteriological and clinical studies of, 13

- Kyrine fraction obtained on partial hydrolysis of proteins, 142
- L** A FORGE, F. B. See LEVENE and LA FORGE, 134
- Lactalbumin, gliadin, and the protein of the rice kernel, some products of hydrolysis of, 335
- LEAVENWORTH, CHARLES S. See OSBORNE, VAN SLYKE, LEAVENWORTH, and VINOGRAD, 335
- Lesions, pneumonic, production of, by intrabronchial insufflation of unorganized substances, 132
- Lesions produced by arsenicals and their bearing on the problem of specific arsenic therapy, 1
- Lethal dose of strophanthin, action of, in normal animals and in animals infected with pneumonia, 438
- Leukemia and pseudoleukemia, chronic, bacteriological results in, 123
- LEVENE, P. A. The relation between the configuration and rotation of epimeric monocarboxylic sugar acids, 154
- LEVENE, P. A., and LA FORGE, F. B. On *d*-lyxohexosaminic acid and on  $\alpha$ - $\alpha$ -anhydro-mucic acid, 134
- LEVENE, P. A., and MEYER, G. M. On the action of aseptic tissue on glucose, 139
- LEVENE, P. A., and VAN DER SCHEER, J. On the kyrine fraction obtained on partial hydrolysis of proteins. Second communication, 142
- LEVENE, P. A., WEST, C. J., ALLEN, C. H., and VAN DER SCHEER, J. Synthesis of normal tridecylic and tetracosanic acids, 149
- LEVIN, ISAAC, and VAN SLYKE, DONALD D. Results of applying a quantitative method to the Abderhalden serum test for cancer, 321
- LOEB, JACQUES. Calcium in permeability and irritability, 238
- . On the rôle of electrolytes in the diffusion of acid into the egg of *Fundulus*, 218
- . Rules and mechanism of inhibition and correlation in the regeneration of *Bryophyllum calycinum*, 165
- . The salts required for the development of insects, 246
- LOEB, JACQUES, and CATTELL, McKEEN. The influence of electrolytes upon the diffusion of potassium out of the cell and into the cell, 193
- LOEB, JACQUES, and CHAMBERLAIN, MARY MITCHELL. An attempt at a physicochemical explanation of certain groups of fluctuating variation, 229
- LOEB, JACQUES, and WASTENEYS, HADOLPH. Note on the apparent change of the osmotic pressure of cell contents with the osmotic pressure of the surrounding solution, 224
- LOSEE, J. R. See VAN SLYKE, VINOGRAD-VILLCHUR, and LOSEE, 458
- d*-Lyxohexosaminic acid and  $\alpha$ - $\alpha$ -anhydro-mucic acid, 134
- MC**LEAN, FRANKLIN C. The numerical laws governing the rate of excretion of urea and chlorides in man. II. The influence of pathological conditions and of drugs on excretion, 285
- MELTZER, S. J. See KLINE and MELTZER, 132
- Meninges, effect of irritation on permeability of, for salvarsan, 267
- Metabolic studies in diabetes, 325
- Metabolism and nutrition in infants, V, 308
- Method for analysis of proteins by determination of chemical groups characteristic of different amino-acids, 356
- Method, micro-, for gasometric determination of aliphatic amino nitrogen, 486
- Method, quantitative, results of applying, to the Abderhalden serum test for cancer, 321
- MEYER, G. M. See LEVENE and MEYER, 139
- Micro-method for gasometric determination of aliphatic amino nitrogen, 486
- Microorganisms *in vitro* and type strains of pneumococci *in vitro* and *in vivo*, action of ethylhydrocuprein (optochin) on, 250
- Milk, protein (*Eiweissmilch*), composition and preparation of, 308
- Milk, woman's, chemical study of, especially its inorganic constituents, 380
- Monocarboxylic epimeric sugar acids, relation between configuration and rotation of, 154
- MOORE, HENRY F. The action of ethylhydrocuprein (optochin) on type strains of pneumococci *in vitro* and *in vivo*, and on some other microorganisms *in vitro*, 250



- The chemoserothapy of experimental pneumococcal infection, 368
- A further study of the bactericidal action of ethylhydrocuprein on pneumococci, 421
- MORTON, JOHN J. See MURPHY and MORTON, 128
- MURPHY, JAMES B., and MORTON, JOHN J. The effect of Roentgen rays on the rate of growth of spontaneous tumors in mice, 128
- N**ERVES, peripheral, in transplantation, biology of, 156
- Nitrogen, aliphatic amino, micro-method for gasometric determination of, 486
- NOGUCHI, HIDEYO, and COHEN, MARTIN. Bacteriological and clinical studies of an epidemic of Koch-Weeks bacillus conjunctivitis associated with cell inclusion conjunctivitis, 13
- Nutrition and metabolism in infants, V, 308
- O**PTOCHIN (ethylhydrocuprein), action of, on type strains of pneumococci *in vitro* and *in vivo*, and on some other microorganisms *in vitro*, 250
- OSBORNE, THOMAS B., VAN SLYKE, DONALD D., LEAVENWORTH, CHARLES S., and VINOGRAD, MARIAM. Some products of hydrolysis of gliadin, lactalbumin, and the protein of the rice kernel, 335
- Osmotic pressure of cell contents, apparent change of, with osmotic pressure of surrounding solution, 224
- P**ATHOGENY of arthritis in rheumatic fever, 109
- Pathological action of arsenicals on the adrenals, 93
- Pathological conditions and drugs, influence of, on excretion, 285
- PEARCE, LOUISE, and BROWN, WADE H. Chemopathological studies with compounds of arsenic. I. Types of the arsenic kidney, 75
- Chemopathological studies with compounds of arsenic. II. Histological changes in arsenic kidneys, 83
- PEARCE, LOUISE. See BROWN and PEARCE, 93
- Peripheral nerves in transplantation, biology of, 156
- Permeability and irritability, calcium in, 238
- Permeability of meninges for salvarsan, effect of irritation on, 267
- Physicochemical explanation of certain groups of fluctuating variation, 229
- Pneumococcal infection, experimental, chemoserothapy of, 368
- Pneumococci, bactericidal action of ethylhydrocuprein on, 421
- Pneumococci, biologic classification of, 489
- Pneumococci, type strains of, action of ethylhydrocuprein (optochin) on, *in vitro* and *in vivo*, and on some other microorganisms *in vitro*, 250
- Pneumococcic septicemia in rabbits, method of serum treatment of, 49
- Pneumonia, action of lethal dose of strophanthin in normal animals and in animals infected with, 438
- Pneumonic lesions, production of, by intrabronchial insufflation of unorganized substances, 132
- Potassium, influence of electrolytes upon diffusion of, out of the cell and into the cell, 193
- Protein milk (*Eiweissmilch*), composition and preparation of, 308
- Protein of the rice kernel, gliadin, and lactalbumin, some products of hydrolysis of, 335
- Proteins, analysis of, improvements in method for, by determination of chemical groups characteristic of different amino-acids, 356
- Proteins, kyriine fraction obtained on partial hydrolysis of, 142
- Pseudoleukemia and leukemia, chronic, bacteriological results in, 123
- Q**UANTITATIVE method, results of applying, to the Abderhalden serum test for cancer, 321
- R**AYS, Roentgen, effect of, on rate of growth of spontaneous tumors in mice, 128
- Reaction, Abderhalden, 458
- Regeneration of *Bryophyllum calycinum*, rules and mechanism of inhibition and correlation in, 165
- Rheumatic fever, pathogeny of arthritis in, 109
- Rice kernel, protein of, gliadin and lactalbumin, some products of hydrolysis of, 335
- Roentgen rays, effect of, on rate of growth of spontaneous tumors in mice, 128

- S**ALTS required for development of insects, 246
- Salvarsan, effect of irritation on permeability of meninges for, 267
- Septicemia, pneumococcic, in rabbits, method of serum treatment of, 49
- Serum, antipneumococcus, mechanism of curative action of, 40
- Serum test for cancer, Abderhalden's, results of applying a quantitative method to, 321
- Serum treatment of pneumococcic septicemia in rabbits, method of, 49
- Spontaneous tumors in mice, effect of Roentgen rays on rate of growth of, 128
- STILLMAN, EDGAR, and SWIFT, HOMER F. The effect of irritation on the permeability of the meninges for salvarsan, 267
- Strophanthin, action of lethal dose of, in normal animals and in animals infected with pneumonia, 438
- Sugar acids, epimeric monocarboxylic, relation between the configuration and rotation of, 154
- SWIFT, HOMER F. See STILLMAN and SWIFT, 267
- T**EST, Abderhalden's serum, for cancer, results of applying a quantitative method to, 321
- Tetracosanic and tridecylic normal acids, synthesis of, 149
- Tissue, aseptic, action of, on glucose, 139
- Transplantation, biology of peripheral nerves in, 156
- Tridecylic and tetracosanic normal acids, synthesis of, 149
- Tumors, spontaneous, in mice, effect of Roentgen rays on rate of growth of, 128
- Typhoid bacilli injected intravenously into normal rabbits, fate of, 58
- U**REA and chlorides, numerical laws governing rate of excretion of, in man, 285
- Urine and feces of male infants, a comfortable arrangement for separate collection of, 505
- V**AN DER SCHEER, J. See LEVENE and VAN DER SCHEER, 142
- See LEVENE, WEST, ALLEN, and VAN DER SCHEER, 149
- VAN SLYKE, DONALD D. Improvements in the method for analysis of proteins by determination of the chemical groups characteristic of the different amino-acids, 356
- Note on the micro-method for gasometric determination of aliphatic amino nitrogen, 486
- VAN SLYKE, DONALD D., VINOGRAD-VILLCHUR, MARIAM, and LOSEE, J. R. The Abderhalden reaction, 458
- VAN SLYKE, DONALD D. See LEVIN and VAN SLYKE, 321
- See OSBORNE, VAN SLYKE, LEAVENWORTH, and VINOGRAD, 335
- VINOGRAD, MARIAM. See OSBORNE, VAN SLYKE, LEAVENWORTH, and VINOGRAD, 335
- VINOGRAD-VILLCHUR, MARIAM. See VAN SLYKE, VINOGRAD-VILLCHUR, and LOSEE, 458
- W**ASTENEYS, HARDOLPH. See LOEB and WASTENEYS, 224
- WEST, C. J. See LEVENE, WEST, ALLEN, and VAN DER SCHEER, 149
- WOLLSTEIN, MARTHA. An immunological study of *Bacillus influenzae*, 28
- Y**ATES, J. L. See BUNTING and YATES, 123



